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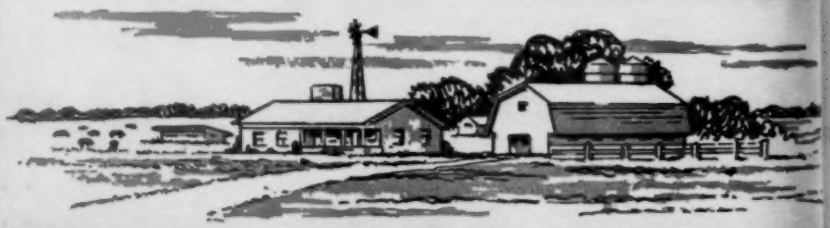

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DEVELOPMENT AND TRANSMISSION OF TOBACCO RINGSPOT VIRUS IN SOYBEAN [1]

Kirk L. Athow and J. B. Bancroft

SUMMARY

One hundred per cent seed transmission of tobacco ringspot virus, the incitant of the bud blight disease, has been observed in soybean. The efficiency of seed transmission depends on the time of infection of the seed-bearing plants. The virus is associated with the embryonic tissue of the seed but not with the seed coat. Epidemiological studies show that the incidence of the disease can be correlated with the type of crop adjacent to soybean fields. Indirect evidence indicates that insect transmission is responsible for the spread of the disease since the virus is not soil-transmitted and seed transmission under natural conditions does not account for the rapid increase of the disease during epiphytotics.

TRSV (tobacco ringspot virus) causes a very destructive disease commonly called bud blight of soybean (*Glycine max* (L.) Merrill). Its numerous symptoms have been described by Allington (1) Hildebrand and Koch (8), Kahn and Latterell (11). The disease has been most prevalent in the heavy soybean-producing area of the midwestern United States, but it has been sporadic or cyclic in occurrence. Between 1943 and 1947, losses of 25–100% were reported in some fields (10). From 1949 through 1954 the disease was much less prevalent and severe. It again became very serious in many fields in Indiana during 1955–57, but was practically nonexistent in 1958. The heavily infected areas were always adjacent to legume-grass mixtures or other uncultivated grassy areas.

Pierce (15) noted the destructive nature of TRSV on soybean as early as 1934, but did not observe its occurrence in nature. Samson (16) reported finding the disease in experimental plants of vegetable soybeans in Indiana in 1941. Melhus (13) observed it in Iowa in 1942, and Johnson (9) reported the disease on soybean in Ohio in 1943. In 1946 Allington (1) verified earlier identifications of the virus by thermal inactivation tests, cross-protection, and symptomatology. He named the disease "bud blight" and discussed the possibility of insect transmission, because of its occurrence around margins of fields. Until 1954, it was assumed that the virus was not seed-transmitted in soybean. This assumption was based on unpublished results obtained in Illinois and on the work of Hildebrand and Koch (8), in Ontario, Canada. The latter found 0.52% infection among plants grown from seed obtained from infected plants, compared with 0.15% infection among plants grown from general-run seed and seed from virus-free plants. In 1954, Desjardins et al (5) reported 78 and 54% transmission through seed from artificially inoculated plants. The lower infection in the second test was attributed to a higher greenhouse temperature. In 1957, Dunleavy (6) reported 1.2% transmission of TRSV from soybean to soybean by the differential grasshopper (*Melanoplus differentialis* Thos.), but concluded that it could not be considered an important vector, because of its feeding habits and the manipulations necessary to obtain even this low infection. The lack of information on disease development and its relation to seed, soil, or insect transmission prompted the studies reported here.

TABLE 1.—*Effect of adjacent crop on the development and percentage of tobacco ringspot virus infection in soybean*

Date of observation	% infection adjacent to [a]			
	Permanent pasture	Pasture-feed lot	Meadow [b]	Corn
July 16	4	6	1	0.4
July 24	74	32	27	20
August 1	85	45	36	22
August 7	91	57	39	25
August 13	95	61	41	28
September 27	100	70	46	44

[a] Average of 1600 plants per location.
[b] Second-year alfalfa, red clover, timothy.

DISEASE DEVELOPMENT.—On June 8, 1957, 8 rows of Harosoy soybeans were planted around the edge of a field in which soybeans had been heavily infected with TRSV the previous year. Thus, the soybeans were adjacent to a permanent pasture; a pasture-feed lot; a second year meadow of alfalfa, red clover, and timothy; and corn. The first bud blight symptoms (chlorosis of the unifoliate leaves, mottling of the young trifoliate leaves, or bronzing, curling, and brittleness of the apical growing point) were observed on July 16. On this date, all infected plants in a 200-plant segment of each of the 8 rows on the 4 sides of the field were tagged. Newly infected plants were tagged each week from July 16 to August 13 as symptoms appeared. All subsequently infected plants were tagged on September 27, when the leaves on the uninfected plants were beginning to turn yellow as a result of normal ripening. Throughout these studies the virus was identified by cross-protection tests on tobacco against TRSV[2] and serologically by the ring-precipitin technique against TRSV antiserum. The data in Table 1, based on the presence of symptoms, show a very rapid in-

[1] Accepted for publication April 28, 1959.
Contribution from the Department of Botany and Plant Pathology, Purdue University Experiment Station, Lafayette, Indiana, and Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture. Journal Paper No. 1390, Purdue University Agricultural Experiment Station.

[2] The writers wish to thank Dr. R. W. Fulton for the isolate of TRSV and Dr. E. L. Moorhead for the TRSV antiserum.

TABLE 2.—*Effect of age at the time of infection with tobacco ringspot virus on soybean growth and development*

Date and age of plants when symptoms observed							Percentage of pods with indicated number of seeds				
Date	Age (days)	Height (in.)	Barren plants (%)	Pods per plant (no.)	Seeds per plant (no.)	Seeds per plant (g.)	0	1	2	3	4
July 16	38	13	41	4	5	1.0	10	56	23	11	0.0
July 24	46	15	24	6	11	2.2	3	41	39	17	0.1
August 1	52	17	14	11	19	3.2	7	33	39	21	0.2
August 7	58	19	6	17	31	5.1	5	31	41	23	0.3
August 13	64	19	5	17	32	5.2	4	29	41	26	0.2
September 27	109	21	0.7	21	40	6.8	2	31	43	24	0.2
October 8 (Uninfected ripe)	120	29	0.0	49	102	16.5	0.6	22	46	31	0.4

crease in the percentage of infected plants between July 16 and July 24, during which time infection increased 70% adjacent to the pasture and 20–26% at the other 3 locations. Increase in the percentage of infection during the rest of the season was gradual except for a 16% increase adjacent to corn between August 13 and September 27, which accounted for over one third of the total infection in this area. Each of the 8 rows at the 4 locations had about the same percentage of infected plants. The highest infection, 100% on September 27, was adjacent to the pasture, and the lowest, 44%, adjacent to the corn. Most of the infection apparently took place during a relatively short period, and infection was favored more by proximity to long-established grassy and weedy areas than to corn or a more recently established grass-legume mixture. These data do not reveal the exact time of infection, since the period between infection and symptom expression in nature is not known. However, it probably does not vary greatly from the 7–10 days required for symptom expression in artificially inoculated soybean plants.

The infected and uninfected plants were cut at the soil line, and various agronomic characteristics were measured. The data summarized in Table 2 show a direct relationship between time of infection and plant height, barren plants, pods per plant, and seed per plant. Early infection resulted in less than half the normal height, 41% barren plants, an average of only 4 pods (mostly 1-seeded), and 1 gram of seed per plant. Progressively later infection resulted in greater height, number of pods, and weight of seed per plant, and a smaller percentage of barren plants. Plants showing symptoms of infection at 38 days of age yielded only 6% of normal, and even plants infected after 64 days of age yielded only 40% as much as uninfected plants.

SEED TRANSMISSION.—Infected Harosoy plants were harvested in 1956 when the uninfected plants were ripe. The seeds were bulked, and about 400 plants were grown from them in individul 4-in. pots in the greenhouse soon after harvest and again the following June. Ten per cent of the seedlings were infected with TRSV in each test. This demonstrated a seed transmission from naturally infected plants and showed that the virus remains infectious in the seed for at least 9 months under ordinary storage conditions.

The infected plants in the first test were grown to maturity, and 14 produced at least one seed. The seeds from each plant were planted in single-plant rows in the field. The results (Table 3) show that 47 of the 48 plants that grew were infected with TRSV. Seed from virus-free plants produced no infected plants, and only 2 plants were infected of 249 grown from commercial seed in check rows interspersed throughout the plot.

Seed from each individual infected plant and a few of the uninfected plants were planted in the greenhouse. There were enough seeds to plant 20 seeds from most of the plants. Distribution of these plants by percentage of transmission to their progenies is shown in Table 3. Twenty-eight of the 47 infected plants produced 100% infected progenies; 7, 90–99%; 10, 80–89%; and 2, 60–69%. The over-all average of seed transmission was 93%. The 1 uninfected plant as well as the virus-free and check plants showed no seed transmission to their progenies. This experiment was followed through another generation, with similar results.

Effect of time of infection on seed transmission.—The effect of the time of infection (the age when infection was observed) on seed transmission was studied by planting 10 seeds from each of 70 plants that had shown symptoms at the various dates of observation listed in the section on disease development. The results are presented graphically in Fig. 1. Seventy-eight per cent of the plants showing symptoms at 38 days of age gave seed transmission, and 91% of the seeds on these plants were infected, as compared with 54% infected seed on 15% of the plants that developed symptoms at 46 days of age. Between 46 and 64 days of age the plants showing transmission averaged 13%, and transmission by these plants averaged 10%, but there was a tendency for both percentage of plants showing transmission and percentage transmission to decrease during the period. The data indicate a slight increase in the percentage of plants showing transmission and the percentage transmission by these plants between 64 and 109 days of age, which undoubtedly reflects an error in classification since any infected plants previously overlooked, no matter when they became infected, fell into this group. A definite relationship is shown between the amount of seed

TABLE 3.—*Percentage transmission of tobacco ringspot virus through 2 generations of soybean seed*

First-generation plant number	No. plants in 2nd generation from 14 infected 1st-generation plants [a]		Number of 2nd-generation plants with indicated percentage transmission to their progenies (3rd-generation) [b]						Average % seed transmission to 3rd generation
	Grown	Infected	0	60–69	70–79	80–89	90–99	100	
1	9	9				3	1	5	93
2	3	3				1	1	1	94
3	1	1						1	100
4	1	1						1	100
5	1	1				1			81
6	1	1						1	100
7	12	12		1		1	4	6	94
8	2	2						2	100
9	4	4		1		3			81
10	3	3				1		2	95
11	6	5	1				1	4	83
12	1	1						1	100
13	3	3						3	100
14	1	1						1	100
Total	48	47	1	2	0	10	7	28	93

[a] 40 seeds from each of 21 virus-free plants produced no infected plants, and only 2 of 249 check plants grown from commercial seed became infected.

[b] 23 virus-free plants showed no transmission to their progenies.

transmission and the time of infection or symptom expression.

Location of virus in the seed.—If the virus associated with seed transmission were located on the seed coat, control of seed transmission should be relatively easy. In two experiments, 400 virus-carrying seeds were soaked 1 minute in 10% Na_3PO_4 and the amount of seed transmission was compared with that found with an equal number of unsoaked seeds. No reduction in seed transmission occurred, but the Na_3PO_4 treatment did reduce emergence. It had been previously shown that treating expressed sap from virus-containing plants 1 minute with Na_3PO_4 would completely inactivate the virus. These results indicate that the virus was probably not carried on the seed coat. A further experiment, in which extracts from equal weights of seed coat and embryo (cotyledon and hypocotyl-epicotyl) from infected seeds were assayed on cowpea, showed that the virus was associated with both cotyledon and hypocotyl-epicotyl of the embryo, but not with the seed coat.

SOIL TRANSMISSION.—No evidence was found of soil transmission in studies involving planting soybeans in soil obtained from fields with 100% of the plants infected, or replanting in these fields the following year. Observations indicate that fields with heavy infection one year do not necessarily have heavy infection the following year if all surrounding fields are cultivated. Bud blight has been found to occur in the same fields for several years only when they are next to an undisturbed grassy area, even though this may be no more extensive than a grassy roadside or a wide fence row.

INSECT TRANSMISSION.—Limited studies were made on insect transmission. Except for surveys to determine the prevalence of various insects in and around bud-blight-infected fields through the season, the insect transmission work was done during the summer of 1958 when practically no infected soybean plants were present in the field. All insects collected in soybean fields and in surrounding grassy areas were allowed to feed on soybean plants, either with or without previous feeding on artificially infected soybean plants. Only 2 of more than 1800 plants exposed to insect feeding became infected. The fact that the disease did not develop in the Midwest during 1958, and the failure to transmit the virus from artificially infected plants by the insects collected, suggests that the insect or insects capable of transmitting the virus were not present.

DISCUSSION.—Transmission of TRSV through seed from naturally infected soybean plants has not been

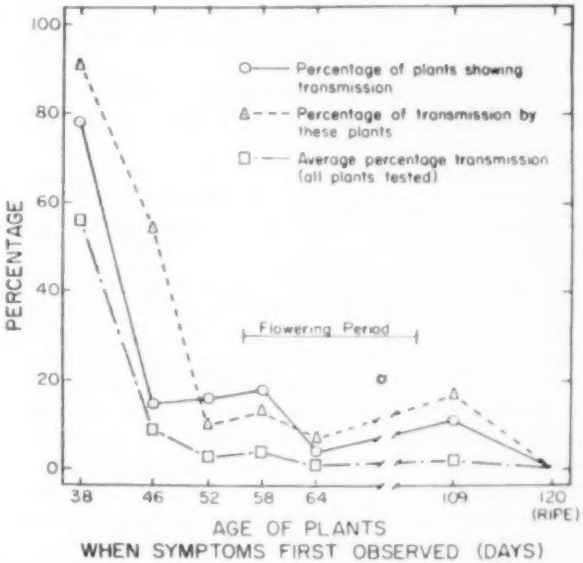

Fig. 1. The relation between the age of soybean plants when TRSV symptoms were first observed and the percentage of TRSV transmitted through the seed.

reported previously. The fact that the plants must become infected early in their development for seed transmission to result probably accounts for the failure of earlier workers to demonstrate seed transmission.

From other studies in 1958[3] it was found that the Harosoy soybean variety used in these tests begins to flower when about 56 days old, with the flowering period averaging 34 days. Therefore, the relation shown between the amount of seed transmission and the time of infection or symptom expression was probably a relation between seed transmission and flowering, or more precisely, fertilization. Kato et al (12) observed that embryo-sac formation in soybeans is completed 3 days before flowering and fertilization, which occur at the same time. Nelson (14) reported that seed from plants infected after flowering rarely transmit the virus of bean mosaic, and Crowley (4) demonstrated that seed transmission of common bean mosaic virus depends on the ability of the virus to infect the microspores, or embryo sac before fertilization. TRSV in soybean then conforms to the theory of Bennett (3) and Crowley (4) that, in order to be seed transmitted, a virus must be able to infect its host systemically, to infect and survive in the haploid gametophytic cells, and to survive in the embryo throughout its development, maturation, storage, and germination. This theory is based on the generally accepted assumptions that there are no plasmodesmatal connections to embryos and that the likely path for movement of virus between cells is by way of the plasmodesmata.

A very high percentage of the seeds on soybean plants grown from infected seed transmitted the TRSV. A review of the literature would indicate that this is apparently peculiar to TRSV of soybean. Crowley (4) stated that percentage of transmission exceeds 50% in only 4 of the 45 viruses reported to be seed transmitted. Bennett (3) stated that there appears to be no instance reported of the transmission of a virus through all of the seeds of an infected plant. TRSV, however, was found to be transmitted by 100% of the seed from over half of the plants grown from infected seeds.

With few exceptions (7, 17, 18), the results of previous seed-transmission studies were based on only one generation and were obtained by determining the number of infected plants resulting from planting seeds grown on naturally infected plants, without regard to stage of development when infected, or on artifically inoculated plants. The latter plants were generally inoculated as early as possible, but even this may not have allowed sufficient time for multiplication and movement of the virus in concentrations to effect maximum infection before fertilization. Of the exceptions that have come to the writers' attention, Snyder (17) reported only 3.3% transmission of cowpea mosaic in commercial asparagus bean seed, as compared with 37% infection among plants grown from infected seeds. Fajardo (7), without presenting experimental data, stated that plants arising from seeds infected with common bean mosaic virus produced more infected seeds than did plants that became infected in the field. Yu (18), however, found no difference in the amount of mosaic in cowpea plants grown from infected seed or from seed from naturally infected plants. In the present studies, transmission was followed through several generations by repeatedly planting seeds from infected plants that had been grown from infected seeds. Therefore, the seedlings were continuously infected and there was a period of about 56 days for virus multiplication and movement prior to fertilization. This is not contradictory to the statement of Crowley (4) that transmissibility is not a property of any virus or of any host, but is clearly an interaction of virus and host. It does suggest, however, that a re-evaluation may reveal a higher percentage of seed transmission of some of the viruses in certain hosts.

There is no obvious explanation why some of the seeds produced on plants grown from infected seeds were apparently virus-free or at least did not transmit the virus. The position of the pod on the plant or the seeds in the pod may have some bearing on the failure to transmit. Fajardo (7), with common bean mosaic virus, found evidence of the latter but not of the former. It is conceivable, even with a systemic virus, that the virus may not be in sufficient concentration in the nucellar and anther tissues over the entire plant to achieve 100% infection. Kato et al (12) described in detail the ontogeny and interrelationships of the soybean seeds in the pod from the time of ovary differentiation to seed maturation. They showed that unfavorable conditions, such as lack of moisture, can alter the normal pattern of seed development in a manner that tends to favor development of the apical seed (farthest from the pedicel.) If one assumes that infection with TRSV has a similar effect, then the seeds that often do not develop on infected plants may be the central and basal ones. If these seeds happen to develop, their position for infection may be less favorable than that of the apical seed. Studies are now being made to attempt to correlate seed position with seed transmission.

Seed transmission is probably of little importance in the development of the bud blight disease of soybean, because of the small amount of seed produced on early-infected plants or plants that develop from infected seeds. Seed transmission, however, could serve to perpetuate and disseminate the virus. For this reason, it is advisable to rogue all infected plants from seed fields.

Since the virus is not soil-transmitted and seed transmission does not account for the rapid build-up of the disease, evidence still points to insect transmission as the primary method of the spread. It was recently found (2) that several biennial and perennial species of plants commonly found in uncultivated areas are natural symptomless hosts of TRSV. The rapid build-up of the disease adjacent to uncultivated areas in which naturally infected perennial plants may serve as a perpetual virus reservoir lends support to the theory of insect transmission. The cyclic and sporadic

[3] Unpublished data kindly furnished by Dr. A. H. Probst.

occurrence of the disease suggests that the vector is unable to overwinter in some years, or that its numbers are so reduced by other biological factors that several years are required for it to become re-established. It is possible that the soybean is not the preferred host of the vector, and, in years of adequate moisture, as in 1958, when uncultivated areas do not dry up, the insects do not migrate into soybean fields. However, if this were the case, some infection, rather than the absence of infection, would be expected in soybean plants along the margin of fields, because of their proximity to infected perennial plants.

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A TEMPERATURE-RELATIVE HUMIDITY INDEX FOR PREDICTING THE OCCURRENCE OF BACTERIAL SOFT ROT OF IRISH POTATOES[1]

J. B. Kendrick, Jr., Randolph T. Wedding, and Albert O. Paulus

SUMMARY

A temperature-relative humidity index calculated from the formula $X = \frac{2.5\ (\%\ \text{RH-hrs. above } 90\%) + (^\circ\ \text{hrs. above } 40^\circ\text{F})}{100}$ was found to have a positive significant correlation to the incidence of bacterial soft rot in White Rose potato tubers. Application of the index, in the light of knowledge concerning conditions favoring suberization and wound periderm formation in tubers, allows the prediction of the occurrence of soft rot and measures the efficiency of systems designed to dry the washed and scrubbed potatoes. Naturally infected potato tubers stored at approximately 100% RH and temperatures of 47°, 64°, and 68°F developed visible symptoms of the disease in increasing amounts as the storage temperature was increased. By extrapolation, the line of best fit indicates that tubers stored at 40°F, even though at 100% RH, will not develop soft rot during the first 26 hours. At temperatures from 60–70°F, relative humidities of 90% or less favor suberization and wound periderm formation more than soft rot development and thus are likely to be responsible for preventing or checking the disease.

The practice of brushing and washing tubers of certain potato varieties, particularly White Rose, in preparing them for market has created an added hazard to delivery of a sound product to the buyer. Under some shipment conditions, washed tubers have shown a greater tendency than unwashed ones to develop soft rot incited by *Erwinia carotovora* (L. R. Jones) Holland. The importance of free moisture on the tubers and high relative humidity during shipment have for many years been recognized as major factors in encouraging the development of soft rot.

Factors affecting the development of bacterial soft rot of potatoes have been the subject of many investigations and reports. Nearly all have shown that tem-

[1]Accepted for publication April 23, 1959.
Paper No. 1126, University of California Citrus Experiment Station, Riverside, California.

perature and moisture are of cardinal importance in decay development. For instance, a report from Rhode Island (1) states that in wet soils tubers will rot at soil temperatures as low as 40°F, but progress of the disorder is much faster at 65–75°F. Davidson (3) reported that lenticel infection of tuber initials required 96 hours at temperatures of 40° and 65°F, but only 24 hours at 102°F when grown in saturated soils.

Ruehle (10), in 1940, reported that new tubers were very susceptible to bacterial soft-rot infection of lenticels in waterlogged soils and that washing infected tubers with 1% solutions of sodium hypochlorite did not prevent further decay. His extensive study of ways to control this disease in washed potatoes showed that washed and scrubbed tubers should be dried in heated air before packaging. Ramsey et al (7) and Rose (8) subsequently reported that early potatoes are more susceptible to this disease when they are washed and shipped wet. Nielsen (5) pointed out that potatoes decreased in susceptibility to soft-rot decay as they aged when they were stored at 61°F, whereas there was very little difference in their susceptibility when stored at 85°F. Both storage conditions were at high relative humidities. Jones and Dowson (4) reported that temperatures of 41–50°F and a relative humidity of 86% favored the formation of a bacterial retarding barrier in the tubers and accounted for the decreased tuber susceptibility under these conditions.

Smith and Ramsey (11) in a comprehensive report on bacterial lenticel infection of early potatoes studied the influences of temperature and humidity upon infection. Their humidities were developed over mixtures of sulfuric acid and water and covered a range from 70.4 to 100%. Below 72°F and at relative humidities less than 94.8%, visible lenticel infection did not develop within 72 hours. In general, the higher the temperature and humidity the more quickly the infection became apparent.

It is clear from these reports that temperature and moisture are intimately associated with soft-rot development both in the soil and in storage or transit.

The work reported herein is an amplification of Smith and Ramsey's investigations (11) and shows the incidence of lenticel infection by *E. carotovora* in stored and/or shipped potatoes is a function of a mathematically expressed relationship between time, temperature, and relative humidity (RH).

Methods.—U. S. #1 fall-grown White Rose potatoes harvested in February and March were used in all tests. The potatoes were taken from the end of the grading table in a commercial packing shed after they had been washed and scrubbed; they were then divided into 100-lb lots and placed in the commercial-type burlap shipping sack. Several preliminary trials had shown that the particular potato source used in these studies developed a high incidence of natural lenticel infection by *E. carotovora* followed by decay of the tubers when they were stored at approximately 70°F and 100% RH. The test lots were brought into the laboratory within 30 minutes after they were selected at the packing shed, and temperature treatments were started immediately.

Large constant-temperature rooms maintained at 50°, 68°, and 78°F were used to study temperature effects upon lenticel infection. To hasten the establishment of 3 temperature differentials for the different tuber lots, each 100-lb. lot was immersed in water at the respective storage room temperatures for 1 hour.

Preliminary trials formed the basis for the experiment reported here to determine the effect of RH, temperature, and time upon soft-rot development. Tubers at high relative humidity were sacked after temperature equilibration in wet sacks, and those at low RH were dried with toweling and placed in dry sacks. The wet sacks were sprayed with water as needed to maintain a highly humid atmosphere. At each of the 3 temperatures, therefore, there were two 100-lb. lots of potatoes, one lot in a dry sack and one in a wet sack.

Relative humidity was determined by wet- and dry-bulb temperature differences measured by thermocouples placed in the center of each sack. It was recognized that this measurement gave only a near approximation of the actual RH because it was not possible to provide the standard air movement past the wet bulb required for precise RH determination. The dry-bulb temperature was utilized for estimating the actual temperature of the storage treatment.

These measurements were recorded on a multiple point Leeds and Northrup recorder at 45-minute intervals for 26 hours. At the end of this period all the potatoes in each of the 6 sacks were examined and the incidence of bacterial lenticel infection was determined.

A second experiment, also based upon previous trials, was conducted simultaneously and set up to determine the rate of development of bacterial lenticel infection at approximately 100% RH at 3 different temperatures. Three additional lots of approximately 100 U. S. #1 potato tubers each were secured at the same time and place as those used in the experiment just described. Each lot was returned to the laboratory and placed immediately in one of the water baths previously brought to the temperature of the room

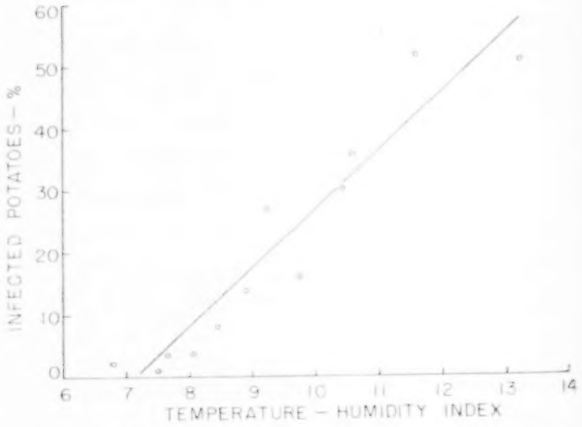

Fig. 1. Correlation of the temperature-relative humidity index with the incidence of bacterial soft rot in potato tubers.

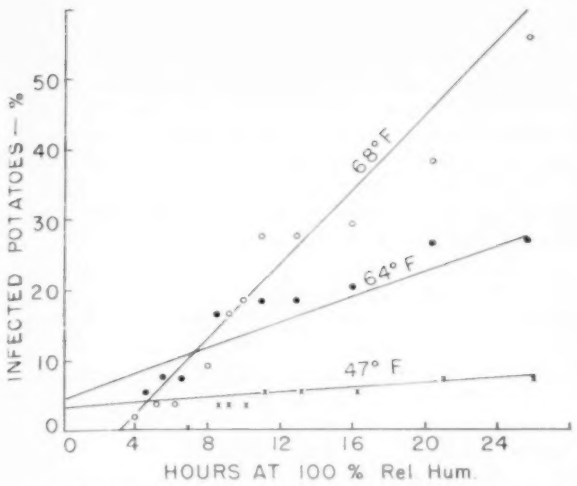

Fig. 2. Correlation of the incidence of bacterial soft rot in potato tubers stored at 100% RH for varying lengths of time at different temperatures.

where the potatoes were to be stored (50°, 68°, and 78°F). After 1-hour immersion the wet tubers were placed in wet sacks together with dry- and wet-bulb temperature-recording thermocouples. All the tubers were examined for lenticel infection at frequent intervals starting 4 hours after the desired temperature was attained. The tubers and sacks were moistened as needed to maintain approximately 100% RH for the duration of the experiment.

RESULTS.—Fig. 1 presents results of the experiment designed to show the relationship of temperature, RH, and time to bacterial lenticel infection. The percentage of visible infection is shown on the Y-axis, and the temperature-humidity index is shown on the X-axis. Since RH is affected by temperature and both have been shown by a number of investigators to influence rate and incidence of soft-rot infection, the data (partially shown in Table 1) were subjected to multiple regression analysis to assess the role of each. For this analysis a base of 90% RH and 40°F was selected by virtue of trial analyses, which showed that these values gave the best approximation at the cut-off points for influence of RH and temperature on infection. Since visible infection is also a function of time, integrated values of degree F-hours and per cent RH-hours were used as the X_1 and X_2 variables in the multiple regression equation. For any given period, the per cent RH-hour integer is the sum of all differences between 90% and each hourly mean RH greater than 90%. Correspondingly, the temperature-time integer is the sum of all differences between 40°F and each hourly mean temperature greater than 40°F.

When X_1 = degree-hour differences above 40°F, and X_2 = per cent-hour differences above 90%, the equation for the multiple regression is

$$Y = -65.57 + 0.0925X_1 + 0.2283X_2$$

The multiple regression coefficient R = 0.9497 indicates a very close fit to the regression plane and a high degree of significance for the relationship of temperature, RH, and time. It will be noted that the coefficient of X_2 is approximately 2.5 times as great as that of X_1. This means that for every degree change in temperature there must be approximately a 2.5 unit change in RH to exert an equal effect. Thus, in order to show a straight-line relationship this factor (2.5) is incorporated into the formula for the temperature-humidity index. When this is done, the index (X) is calculated from the following formula:

$$X = \frac{2.5\ (\%\ \text{RH-hrs. above } 90\%) + (^\circ\ \text{hrs. above } 40^\circ\text{F})}{100}$$

The divisor (100) is introduced into the formula so that the values may be more easily handled in calculations.

When the index values (X) are correlated with the incidence of lenticel infection (Y), fitting the data by the method of least squares indicates that the line of best fit has the formula: $Y = -65.65 + 9.21X$ (Fig. 1). The correlation coefficient for this relationship is $r = 0.950$, significant at the 1% level.

Fig. 2 summarizes the results of the experiment designed to show the rate of development of bacterial lenticel infection under conditions of a water-saturated atmosphere at 3 different temperatures. The per cent infected potatoes are shown on the Y-axis, and the hours at 100% RH are shown on the X-axis. Although the potatoes were stored in rooms maintained at temperatures of 50°, 68°, and 78°F, the actual average air temperatures in the sacks were 47°, 64°, and 68°F, respectively.

The increase in incidence of infection with increase in time is positively correlated at each of the temperatures. The lines of best fit were constructed from the equations: $Y = 1.98 + 0.222X$, $Y = 5.11 + 0.843X$, and $Y = -7.24 + 2.393X$ for temperatures of 47°, 64°, and 68°F, respectively. In this formula, X = hours and Y = percentage of infections. The correlation coefficients for these 3 temperatures listed in the same order are: $r = 0.919$, $r = 0.854$, and $r = 0.976$, all of which are significant at the 1% level.

From the slopes of the lines, a calculation of the regression of slope vs. temperature gives the following equation: $Y = -1.7796 + 0.0446X$. Here X = temperature in degrees F and Y = slope of per cent infected tubers vs. time (number of infected tubers per hour). The correlation coefficient of this relationship is $r = 0.963$, which is significant at odds of **19:1**.

TABLE 1.—*Temperature-RH indexes and corresponding infection incidences (%) utilizing field-infected White Rose potato tubers placed at various combinations of temperature (°F) and RH (%) for 26 hours*

Temp. range (°F)	Relative humidity range (%)					
	91–93		94–96		97–100	
	Index	Infection	Index	Infection	Index	Infection
48–50					8.5	8.1
53–55					8.8	13.9
60–62			8.1	3.8	10.4	30.0
63–65	6.7	2.2	9.8	15.1	13.2	50.5
66–68			10.6	35.1		

TABLE 2.—*Theoretical temperature-RH indexes for various hypothetical combinations of temperature (°F) and relative humidities (% RH) for 24- and 48-hour periods. Time, temperature, and RH combinations favorable for cell suberization indicated by (s)*

Temp. (°F)	Relative humidity (%)					
	90	95	100	90	95	100
	24 hours			48 hours		
50	2.4	5.4	8.4	4.8	10.8	16.8
55	3.6	6.6	9.6	7.2	13.2	19.8
60	4.8	7.8	10.8	9.6(s)	15.6(s)	21.6
65	6.0	9.0	12.0	12.0(s)	18.0(s)	24.0
70	7.2(s)	10.2(s)	13.2	14.4(s)	20.4(s)	26.4

When Y = 0, then X = 39.9°F, which means that at temperatures below 40°F and at 100% RH no infection would develop within 26 hours.

Table 1 contains data from several experiments showing index values and subsequent incidence of infection after 26 hours under several combinations of temperature and RH.

Table 2 contains temperature-RH indexes calculated from the index equation for various theoretical conditions of temperature and RH for 24 and 48 hours. Index values greater than 6.0 suggest that soft-rot infections should become visible. With an increase in the index value, there should be an increase in the incidence of visible infection. In Table 2, certain values are followed with "(s)." This designation is placed at combinations of temperatures and time values where, according to Artschwager's data (2), suberization would be likely to occur, followed by periderm formation.

DISCUSSION.—Washed potatoes are often shipped under widely varying conditions of relative humidity and temperature. This is particularly true in regions where potatoes are produced and marketed at different seasons of the year. Potato shippers in these areas have requested information that would enable them to predict conditions favorable for bacterial soft-rot occurrence. A dependable warning system would enable them to use the expensive hot-air drying process only during periods favorable for soft-rot development and thus effect a saving in the cost of processing.

Inquiries have likewise been made concerning the degree of drying necessary to decrease the hazard of soft-rot development. With knowledge of the minimum requirements of temperature and moisture for soft-rot occurrence, it is possible to assess the efficiency of drying systems which endeavor to provide an unfavorable environment for development of the decay.

The mathematical relationship of time, temperature, and relative humidity determined in these studies is useful in supplying information relative to prediction of soft-rot occurrence. Certain qualifications are necessary in the application of the temperature-humidity index, however. The data were developed using the potato variety White Rose. Different potato varieties are reported to have varying susceptibilities to this disease (5). When the index is used for varieties other than White Rose, it should be interpreted in the light of information regarding the comparative susceptibility of White Rose and the other variety.

Data were also developed on fully mature tubers that had not been subjected to excessive skinning, bruising or sunlight. These factors, which have been reported to increase tuber susceptibility to decay (5, 9), would alter the slope of the curve.

Finally, thermophilic strains of the causal organism would not fit precisely in the mathematical relationship presented. With the realization that variables other than temperature and RH affecting pathogenicity and susceptibility may operate, it is not assumed that the temperature-humidity index represents an absolute relationship to soft-rot decay. It does, however, provide a basis for statistical estimation of the probability of soft-rot occurrence under prescribed conditions of the environment. Credibility is given to the index concept by Smith and Ramsey's (11) report that at 100% RH and 75°F, 50% of the White Rose tubers showed symptoms of infection 24 hours after being inoculated. Transformation of these data into the temperature-humidity index gives a value of 13.7, which shows (Fig. 1) that between 50 and 60% of the tubers should decay.

It is evident from the data (Fig. 2) that visible symptoms of soft-rot development can appear rather quickly at temperatures greater than 64°F and at 100% RH. Within 8 hours approximately 10% of the tubers showed infection. This is somewhat earlier than reported by Smith and Ramsey (11), who stated that visible infection developed 18 hours after being inoculated at 85°F and 100% RH. This time-lag difference is probably not as great as it might seem from the two sets of data, because tubers used in our studies were infected at some time prior to the initiation of the temperature and humidity treatments, although there were no visible signs of infection on the tubers when treatments were begun.

The data presented in Table 2 seem to form a sound basis for predicting the development of potato soft rot under varying conditions of temperature and moisture. Although Artschwager's data (2) were developed from potato varieties other than White Rose, they seem to apply also in this case, so there appears to be no great discrepancy between varieties with regard to temperature and humidity requirements for cell suberization and wound periderm formation. Unfortunately, Artschwager does not present information concerning the RH existing during his experiments to determine the influence of temperature upon the time necessary for potato tuber cells to suberize and subsequently develop a wound periderm. He does state, however, that increase in RH from 64% to 95% increased the amount of suberization. Olufsen (6) in his studies on wound periderm formation in potato tubers stated that moderate moisture was essential for periderm formation but that excess moisture caused cell proliferation rather than periderm development. Rose and Schomer (9) reported that moist air favored suberization of tuber cells while dry air retarded its development. Artschwager (2) reported that suberization and wound periderm formation were favored by

an increase in temperature up to 70°F.

From these reports and those concerned with influences of temperature and humidity upon soft-rot development, it is apparent that the development of the barrier to bacterial invasion—cell suberization and periderm formation—as well as soft rot are all favored by elevated temperatures and RH.

This seemingly anomalous situation is clarified by examining the hypothetical situations shown in Table 2 and comparing them with the experimental data shown in Table 1.

At approximately 100% RH the index values and infection incidence increase together with a rise in temperature (Table 1). This is predictable according to the theoretical index values shown in Table 2 for 100% RH and the same temperature range for the same 24-hour period. As temperatures approach 70°F, cell suberization should occur, according to Artschwager after 24 hours. However, as noted by Olufsen (6), excess moisture—presumably approximately 100% RH—would favor cell proliferation rather than suberization so the infection would not be arrested by periderm formation.

At approximately 95% RH the theoretical and actual index values for 24 and 26 hours are in substantial agreement. It will be noted in Table 1, at a temperature of 66–68°F and at RH of 94–96%, that the index value is 10.6 and incidence of infection is 35.1. In Table 2, under approximately similar conditions (70°F and 95% RH), the theoretical index value is 10.2, but suberization should occur at this point. In the experiment, although the infection incidence was high, the development of the lenticel decay was meager and arrested, indicating that periderm formation did occur under these conditions, but at a somewhat slower rate probably because of the slightly lower mean temperature than that reported by Artschwager (2).

The theoretical index values for 48 hours are included in Table 2 to make use of Artschwager's data in conjunction with the temperature-RH index. Cell suberization and periderm formation, according to him, are initiated at 60°F within 48 hours at humidities up to 94%. Therefore, even though index values at temperatures greater than 60°F and relative humidities of 95% or less for 48 hours are large enough to suggest soft-rot occurrence, periderm formation would probably either prevent or arrest soft-rot decay.

In general, it may be noted (Table 1) that soft rot develops at a decreasing rate with RH decreasing from approximately 100% to 90%. From the theoretical values shown in Table 2, it is apparent for a temperature range of 50–70°F that 90% RH is the cardinal point at or below which the disease will probably be arrested or prevented.

The temperature-RH index interpreted in this manner thus forms a useful basis for predicting the occurrence of bacterial soft rot of potato tubers as determined by the incidence of lenticel infection under a variety of storage and transit conditions.

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LYCHNIS RINGSPOT [1]

C. W. Bennett[2, 3]

SUMMARY

A disease characterized by rings and zonate spots on plant leaves was discovered in a plant of *Lychnis divaricata* growing in a greenhouse. The causal virus, transmissible by juice inoculation, produced systemic infection in *Lychnis divaricata, Silene noctiflora, S. gallica, Beta vulgaris, Spinacia oleracea, Callistephus chinensis,* and certain other plants. It produced local lesions in several species in which it did not become systemic. Infected plants of *Tetragonia expansa* and *Stellaria media* produced no seeds, and seed production was drastically reduced in *B. vulgaris, C. chinensis,* and *Silene noctiflora.* The virus was not transmitted by *Cuscuta californica* or by *C. campestris.* No insect vector was found. It was transmitted through a high percentage of seeds of *L. divaricata* and *S. noctiflora* and through lower percentages of seeds of sugar beet, *Cerastium viscosum, Silene gallica,* and probably *Capsella bursa-pastoris,* but not through seeds of *Geranium dissectum,* China aster, or spinach. The virus was also transmitted through pollen of *L. divaricata* and *S. noctiflora.* Flowers of healthy plants of *L. divaricata* pollinated from infected plants produced infected seeds, whereas flowers on the same plants receiving pollen from healthy plants produced virus-free seeds on 9 of 10 plants, and none of the mother plants became infected systemically.

The virus has a thermal inactivation point between 64° and 68°C, retains activity 2–7 days in plant juice at room temperature, and has a dilution end point of about 1:2000. It did not withstand drying in plant juice. It was active in seeds of *L. divaricata* after 992 days of storage. The virus appears to be better adapted to invasion of parenchyma than to invasion of phloem, since it produced local lesions in a number of plants in which it did not become systemic or became only partially systemic. The virus, apparently previously undescribed, is considered to belong to the ringspot group of plant viruses. It differs from other ringspot viruses principally in host range, induced symptoms, and in the low degree of recovery of most of its host plants.

INTRODUCTION.—In June, 1955, a plant of *Lychnis divaricata* Reichenb., grown for use in studies of the sugar beet yellows virus in a greenhouse at the U. S. Agricultural Research Station, Salinas, California, showed a type of ringspot caused by a virus that appears to be different from ringspot viruses previously described. Tests showed that this virus is able to infect a number of plant species, including certain crop plants such as sugar beet and spinach. Principally because of its somewhat unusual relationships to various host plants, the virus was studied further. The results are reported herein. Since the virus was discovered in *L. divaricata* and produced distinct ringspot symptoms on this and other species of *Lychnis,* the disease it causes is designated "Lychnis ringspot," in conformity with usage adopted by the Review of Applied Mycology in applying common names to virus diseases (1).

HOST RANGE OF THE VIRUS.—The host range of the Lychnis ringspot virus has not been studied extensively, but a high percentage of the species tested proved to be susceptible to infection. Local chlorotic or necrotic lesions were produced on inoculated leaves of a number of species that were not infected systemically. In some species, as sugar beet and New Zealand spinach, local lesions were numerous on inoculated leaves but systemic infection occurred in only small percentages of inoculated plants.

Species of plants found susceptible to infection by juice inoculation are listed below, in alphabetical order with respect to family, genus, and species. The virus was not fully systemic in species marked with an asterisk (*); in these species, symptoms were, in most cases, limited to local chlorotic or necrotic spots on inoculated leaves.

AIZOACEAE.—*Tetragonia expansa* Murr., New Zealand spinach.

CARYOPHYLLACEAE.—*Cerastium viscosum* L., sticky mouse-ear chickweed; *C. vulgatum* L., common mouse-ear chickweed; *Gypsophila elegans* Bieb., Gypsophila; *Lychnis chalcedonica* L., Maltese cross; *L. coronaria* (L.) Desr., Mullein pink; *L. divaricata* Reichenb.; *L. haageana* Lem., Haage campion; *Silene gallica* L., windmill pink; *S. noctiflora* L., night-flowering catchfly; *Stellaria media* (L.) Cyr., common chickweed.

CHENOPODIACEAE.—*Atriplex rosea* L., redscale; *Beta patellaris* Moq.; *B. vulgaris* L., sugar beet; **Chenopodium album* L., lamb's-quarters; **C. amaranticolor* Coste & Reyn.; *C. capitatum* (L.) Asch., strawberry blite; **C. murale* L., sowbane; *Spinacea oleracea* L., spinach.

COMPOSITAE.—*Callistephus chinensis* (L.) Nees., China aster.

CRUCIFERAE.—*Capsella bursa-pastoris* (L.) Medic., shepherd's-purse.

GERANIACEAE.—*Geranium dissectum* L., cut-leafed crane's bill.

PRIMULACEAE.—**Samolus parviflorus* Raf., water pimpernel.

SOLANACEAE.—**Capsicum frutescens* L., pepper; **Datura stramonium* L., jimson weed; *Nicotiana clevelandii* Gray; *N. glauca* Graham., tree tobacco; *N. glutinosa* L.; **N. tabacum* L., tobacco var. Samsun; *Petunia hybrida* Vilm., petunia.

It is probable that some of the species showing only

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local infection may be susceptible to systemic infection under certain environmental conditions.

Plants showing no indication of infection include *Lactuca sativa* L. (lettuce), *Nicotiana rustica* L. (wild tobacco), *Phaseolus vulgaris* L. (bean var. Bountiful), *Phytolacca americana* L. (pokeweed), *Lycopersicon esculentum* Mill. (tomato), *Solanum nigrum* L. (black nightshade), *Vigna sinensis* (Torner) Savi (cowpea), and *Zinnia elegans* Jacq. (zinnia).

SYMPTOMS AND EFFECTS OF THE DISEASE.—One of the most characteristic symptoms of Lychnis ringspot is zonate spots or rings on leaves, often showing alternate bands of green and yellow tissue. Spot characteristics and disease severity vary on different plants and on locally and systemically infected leaves.

Symptoms on inoculated leaves.—A high percentage of species of plants tested showed local lesions on leaves inoculated mechanically. On some plants, as sugar beet and Samsun tobacco, lesions were chlorotic and usually continued to spread radially as long as the leaves remained actively growing, often without producing systemic infection. Typical lesions on sugar beet are shown in Fig. 1-A. In other plants, as *Chenopodium amaranticolor*, *C. album* (a broadleaf selection), and *C. capitatum*, lesions soon became necrotic and usually ceased to expand after reaching a diameter of a centimeter or more. In some cases, however, they spread downward along the main veins of leaves, as shown in Fig. 1-B. Effects of the virus on inoculated leaves of plants of *Lychnis divaricata* were often indefinite, although many of the inoculated plants became infected systemically. Definite spots composed of alternate chlorotic and green bands were produced on inoculated leaves of *Lychnis coronaria*. The virus usually continued to spread until the entire leaf was involved.

Symptoms on systemically infected plants.—The range of severity of systemic symptoms was rather wide on different species of plants. *Lychnis divaricata* and *Geranium dissectum* showed little or no dwarfing, and symptoms were largely confined to zonate rings and spots. A leaf of *L. divaricata* with typical severe symptoms is shown in Fig. 1-C. Symptoms were absent from some leaves, possibly because of a tendency toward recovery. However, symptoms sometimes recurred after an apparent partial recovery.

Leaves of *Callistephus chinensis* showed spots and rings of golden yellow tissue, but were not deformed. The flowers were more or less normal in size and shape, but diseased plants were dwarfed.

Leaves of *Silene noctiflora* and sugar beet were markedly deformed and plants were stunted. Leaves of sugar beet plants were straplike and irregular along the margins, and sometimes had rings and lines of chlorotic tissue. A beet plant about 5 months old, infected in the seedling stage, is shown in Fig. 2-A. There was no evidence of recovery in *S. noctiflora* or sugar beet.

The virus rarely became systemic in *Tetragonia expansa*, but the few plants that showed systemic in-

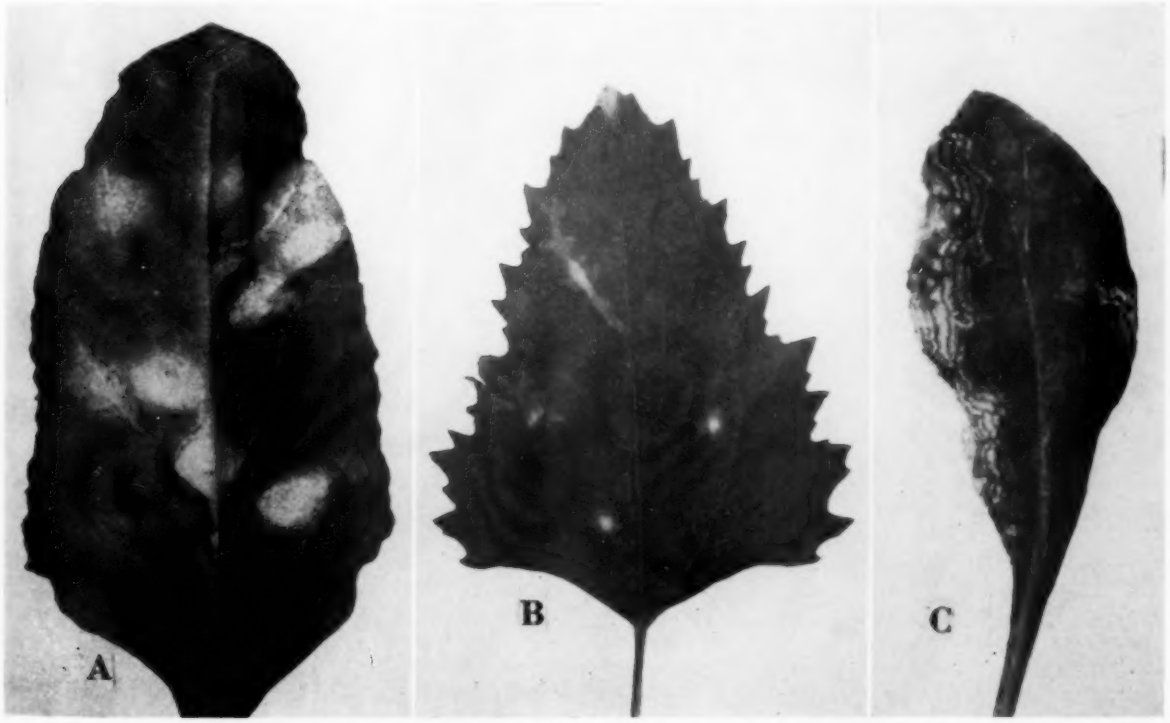

Fig. 1. A) Sugar beet leaf showing local chlorotic lesions produced from mechanical inoculation with Lychnis ringspot virus. Darker areas indicate necrosis. **B)** Leaf of *Chenopodium album* showing local lesions produced from mechanical inoculation, and effects of spread of virus along the veins. **C)** Chronic symptoms of Lychnis ringspot on *Lychnis divaricata*.

fection were severely dwarfed. Stems were small, internodes were shortened, and leaves were less than one-half normal size.

The virus was not completely systemic in Samsun tobacco or petunia. Necrotic lesions were induced on leaves and stems of Samsun tobacco, sometimes widely separated. The character and intensity of symptoms varied greatly on different petunia plants, and even on different shoots of the same plant. In some cases, the virus appeared to invade only limited areas inducing chlorosis or necrosis in otherwise normal parts of the plant. In other instances, the virus appeared to be more completely systemic, and plants produced deformed leaves with various types and patterns of chlorotic tissue. There was no evidence of recovery, though some shoots appeared to escape infection.

Nicotiana clevelandii and *Lychnis coronaria* were the only species that showed clear evidence of recovery. Leaves of *N. clevelandii* showing first symptoms became necrotic and died, but necrosis was absent in new growth. New leaves, however, were deformed and dwarfed and the plants were stunted. Plants of *L. coronaria*, infected in about the 6-leaf stage, showed symptoms after 2 weeks. Zonate spots were produced on 3–5 leaves, but the plants recovered and showed no further evidence of disease.

Effect of the disease on seed production.—One of the most characteristic effects of Lychnis ringspot virus is a reduction in seed yield. Of the species tested, only *Lychnis divaricata*, *Geranium dissectum*, and spinach flowered and fruited normally when infected systemically with the virus. *Stellaria media* produced normal-appearing flowers but set no seeds. Infected *Tetragonia expansa* produced aborted flowers and no seeds. Flowering of infected *Callistephus chinensis* was more or less normal, but more than 90% of the seeds were without germs. The virus was not fully systemic in *Petunia hybrida*. Pods showing rings and

Fig. 2. **A)** Sugar beet plant showing chronic effects of Lychnis ringspot on leaves and flowering stalk. **B)** Portion of fruiting stalk of sugar beet plant infected with Lychnis ringspot virus, showing excessive branching of fruiting stems and clumping of seed balls.

TABLE 1.—*Effect of Lychnis ringspot virus on seed production in Silene noctiflora*

Origin of seeds	Average no. seeds per pod
Diseased plants self pollinated	41.2 [a]
Diseased plants pollinated from healthy plants	64.1
Healthy plants pollinated from diseased plants	12.2
Healthy plants pollinated from healthy plants	148.3

[a] Figure represents average number of seeds in pods with seeds; 91.3% of pods had no seeds.

other lesions produced few seeds, but seed production was apparently unaffected in pods showing no external symptoms. In sugar beet, flowering stalks tended to revert to a vegetative condition and then to send out weak fruiting stems from partially vegetative rosettes. Such stalks produced seed balls sparingly and in clumps, as illustrated in Fig. 2-B. Germination of seeds from infected plants was very much reduced.

The virus apparently affected the production and functioning of both pollen and ovules. Pollen production was reduced drastically in sugar beet and in *Silene noctiflora*. In the latter, most of the stamens on diseased plants failed to elongate, and anthers were sterile. Stamens that elongated had anthers with little pollen.

To obtain information on the relative effects of the virus on male and female gametes, tests were made with *Silene noctiflora* involving the 4 possible crosses of diseased and healthy plants. Pods were harvested and the seeds counted (Table 1). Diseased plants pollinated from diseased plants produced seeds in only 8.7% of the pods counted, and seed production was low in pods that set seeds. Pods of diseased plants pollinated from healthy plants produced fewer than half as many seeds as pods of healthy check plants, indicating that the virus has a marked effect on the ovules. Most of the pods on healthy plants pollinated from diseased plants produced seeds, but seed set was very sparse. The low seed set may have been partly due to difficulty in finding enough pollen in flowers of diseased plants to pollinate the healthy flowers adequately. The results indicate, however, that seed production is reduced if either parent is infected with the virus.

TRANSMISSION.—*Transmission by juice inoculation.*—Lychnis ringspot virus is juice-transmissible by rubbing, but the results often are erratic, ranging from high percentages of infection to slight or none.

Local lesions were produced on most susceptible species by rubbing juice from diseased plants over the surface of leaves sprinkled with carborundum. Often, only a few lesions were produced. Infection appeared to be influenced both by the source of virus and by the plant inoculated. Production of local lesions was most consistent on leaves of *Chenopodium album* (a broadleaf selection), *C. amaranticolor*, and *C. capitatum*. Lesions were evident on leaves of *C. amaranticolor* 42 hours after inoculation. Within 48 hours, they were 1 mm in diameter and in 72 hours they had increased to 2 mm in diameter. Lesions usually reached considerable size, particularly when the affected tissue did not become necrotic.

Tests of insects as agents of transmission.—For more than 2 years there was no evidence of spread of ringspot from diseased to healthy plants under greenhouse conditions. Red mites and thrips, which were present in the houses are apparently not vectors of the virus. In tests with other insects, large numbers were permitted to feed on diseased plants and then transferred, or allowed to move, to healthy seedlings of *Lychnis divaricata*. The species of insects tested were *Circulifer tenellus* (Baker) (beet leafhopper), *Myzus persicae* (Sulz.) (green peach aphid), *Aphis fabae* (Scop.) (bean aphid), and *Rhopalosiphum maidis* (Fitch) (corn leaf aphid). Twenty or more plants were used with each insect, but none showed evidence of infection.

Tests with dodder.—Transmission tests were made with two species of dodder, *Cuscuta californica* Choisy and *C. campestris* Yuncker. Dodder was established on diseased plants of New Zealand spinach and *Lychnis divaricata*, and stems of the parasite were trained to plants of *Chenopodium amaranticolor*, *C. capitatum*, and sugar beet. Twenty healthy plants of each species were parasitized by each species of dodder, but none developed symptoms of disease.

To test the ability of dodder to acquire virus from infected host plants, juice was extracted from stems of *C. californica* growing on an infected plant of New Zealand spinach, and inoculated into plants of *Chenopodium amaranticolor* and *C. capitatum*. Results were compared with those obtained with juice from the infected New Zealand spinach plant on which the dodder was growing. Lesions were not produced in any of the tests with juice from dodder, whereas lesions were numerous in all cases with juice from New Zealand spinach. Further tests showed that the virus was not inactivated in juice of New Zealand spinach by adding an equal amount of juice from dodder. It seems probable, therefore, that dodder is unable to acquire and retain appreciable amounts of virus from infected plants. This may account for the failure of dodder to transmit the virus.

Transmission through seeds.—Lychnis ringspot virus was first obtained from a seedling of *Lychnis divaricata* grown in a screened greenhouse; thus there was evidence of seed transmission of the virus from the beginning of these studies of the virus. After it was found that more than half of the seeds of infected plants of *L. divaricata* may transmit the virus, seeds from infected plants of other susceptible species were tested. The results are in Table 2.

Transmission was greatest in seeds of *Lychnis divaricata* and *Silene noctiflora*. Respective averages of 58.4% and 41.7% of the seedlings from seeds of diseased plants showed definite symptoms. Transmission through seeds was also obtained in *Silene gallica*, *Cerastium viscosum*, and sugar beet, and probably *Capsella bursa-pastoris*. Only 4 of 42 sugar beet seedlings showed infection, but symptoms were evident in the very early stages of development. Cotyledons were narrow, thickened, and distorted, and the true leaves

TABLE 2.—*Results of tests to determine transmission of Lychnis ringspot virus through seeds of different species of plants*

Plant tested	No. seeds planted	No. seedlings obtained	Seedlings infected	
			No.	%
Beta vulgaris		42	4	9.5
Callistephus chinensis	166	93	0	0.0
Capsella bursa-pastoris	100	96	9[a]	9.4
Cerastium viscosum	100	88	24	27.3
Geranium dissectum	150	36	0	0.0
Lychnis divaricata	500	325	190	58.4
Petunia hybrida[b]		419	0	0.0
Nicotiana tabacum[b]		2200	0	0.0
Silene gallica	200	164	28	17.1
Silene noctiflora	100	72	30	41.7
Spinacia oleracea	300	104	0	0.0

[a] Seedlings presumed to be infected since they yellowed and died in the cotyledon stage, whereas seedlings from check lots of seeds remained normal.

[b] Virus was not fully systemic in plants from which seeds were harvested.

were malformed and showed distinct zonate spots. Virus was recovered from the seedlings by transfer to *Chenopodium amaranticolor*. This appears to be the first known instance of transmission of a virus through seeds of sugar beet in the western hemisphere. A number of seedlings of *C. bursa-pastoris* from seeds of diseased plants had deformed cotyledons, and the seedlings died before they produced true leaves. Necrotic lesions were observed on the cotyledons and on the stems. It seems probable that these seedlings were infected but were so sensitive to effects of the virus that they were killed in early stages of growth.

No transmission of the virus was observed through the seeds of *Callistephus chinensis* (China aster), *Geranium dissectum* (cut-leafed crane's-bill), *Petunia hybrida* (petunia), *Nicotiana tabacum* var. Samsun, or spinach. Fewer than 10% of the seeds from infected China aster plants appeared normal. Selected fully developed seeds gave 56% germination, but there was no evidence of seed transmission. It seems probable that invasion of the ovule may have resulted in failure of the embryo to develop, and that viable seeds were produced only from ovules that escaped infection.

The virus was not fully systemic in Samsun tobacco, and flower parts were probably not invaded. The virus was also only partially systemic in petunia, but the seeds tested were collected from pods that showed definite lesions. Symptoms of the disease were relatively mild on plants of *Geranium dissectum* and spinach, and seed production was not appreciably reduced. If the virus passed through the seeds of these species, it was not detected.

Transmission through pollen.—In all cases, where tests were adequate, viruses transmitted through seeds also appear to occur in the pollen of infected plants. Tests were made to determine whether Lychnis ringspot virus is transmitted through pollen of *Lychnis divaricata* and *Silene noctiflora*. Both species show distinct symptoms but produce seeds when infected. In addition, *L. divaricata* is a dioecious species, which greatly simplifies tests of pollen transmission.

In the first test, infected pistillate and staminate plants of *L. divaricata* were placed in one greenhouse compartment, and healthy plants of both types were placed in a second compartment. Crosses were made in the four possible combinations of healthy and diseased plants. Seeds were harvested at maturity, stored through a dormancy period of about 3 months, and planted singly in flats in lots of 100. Counts of diseased seedlings were made at intervals until the plants were about 2 months old.

In tests with *S. noctiflora*, diseased and healthy plants were separated into two groups. Flowers to be pollinated were emasculated in an early stage of anther development, and all other flowers were removed before they opened.

The results are shown in Table 3. The virus was transmitted by pollen to 18.6% of the viable seeds of each species. Pollination of diseased plants with pollen from healthy plants gave more seed transmission than did the reciprocal cross, perhaps indicating either that the virus entered a higher percentage of female gametes or that infected male gametes were at a disadvantage with respect to effecting fertilization. The cross of diseased by diseased plants gave the highest percentage of seed transmission, as might be expected, but the per cent of transmission was not markedly greater than from the cross of diseased by healthy.

Ten healthy pistillate plants of *Lychnis divaricata* were selected for a further study of pollen transmission and of movement of the virus in the mother plant following pollination. On each of the selected plants, 5 flowers were pollinated from a plant with mild symptoms, 5 flowers were pollinated from a plant with severe symptoms, and 5 flowers were pollinated from a healthy plant.

The results, shown in Table 4, indicate a wide range in percentage of transmission by seeds of different lots. There was no indication, however, that pollen from severely affected plants transmitted the virus more frequently than pollen from mildly infected plants. In one of 10 plants, seeds from flowers pollinated from healthy plants transmitted the virus, though this trans-

TABLE 3.—*Transmission of Lychnis ringspot virus through pollen of infected plants of Lychnis divaricata and Silene noctiflora*

Species of plant tested	Cross[a]	Seeds of test plants[b]	
		% germination	% germinated seeds infected
L. divaricata	H × H	86.5	0.0
	H × D	84.9	18.6
	D × H	77.6	30.7
	D × D	74.0	33.6
S. noctiflora	H × H	95.0	0.0
	H × D	64.5	18.6
	D × H	63.0	28.7
	D × D	67.5	37.6

[a] H = uninfected plant; D = infected plant.

[b] Figures represent averages of 100 seeds of each of 5 plants of *L. divaricata* and averages of 100 seeds of two composite lots of seeds of *S. noctiflora*.

TABLE 4.—*Virus transmission through seeds of uninoculated plants of Lychnis divaricata from flowers pollinated from a healthy plant, a plant with mild symptoms, and a plant with severe symptoms*[a]

Plant no.	Pollen from healthy plants		Pollen from plant with mild symptoms		Pollen from plant with severe symptoms	
	% seed germination	% seedling infection	% seed germination	% seedling infection	% seed germination	% seedling infection
1	83	0.0	90	18.9	61	14.8
2	91	0.0	97	5.2		
3	83	0.0	66	7.8	33	36.4
4	78	0.0	82	24.8		
5	90	0.0	87	9.2	86	34.9
6	83	0.0	67	40.3	92	23.9
7	76	0.0	52	26.9	71	16.9
8	77	2.6	70	25.7	83	18.1
9	78	0.0	70	28.6	39	5.1
10	76	0.0	83	44.6	80	17.5

[a] Five flowers on each plant were pollinated from each source; 100 seeds tested from each lot of seeds from each plant.

mission was very low. In 9 of 10 plants, the virus apparently did not move from infected flowers and enter ovules of other flowers of the same plant.

After harvest of the seeds, the tops were removed and the 10 plants were repotted and fertilized to stimulate rapid growth favoring the expression of ringspot symptoms. All the plants remained free of symptoms, and no virus was recovered by inoculation to *Chenopodium amaranticolor*, thus indicating that the virus in the pollen that fertilized the infected seeds did not move out of the tops of the plants and become established in the root system. This appeared to be true even in the plant in which the virus apparently did invade one or more of the ovaries of flowers pollinated with virus-free pollen.

PROPERTIES OF THE VIRUS.—Properties of the Lychnis ringspot virus were determined with juice of infected plants of *Silene gallica* inoculated into plants of *Chenopodium album*. *S. gallica* appeared to supply a rather uniform inoculum, and *C. album* proved to be a satisfactory plant for the production of local lesions from mechanical inoculation.

Thermal-inactivation point.—Juice from infected plants was placed in small, thin-walled test tubes and subjected to the desired temperature in a water bath for 10 minutes. The contents of the tubes were then used to inoculate test plants by rubbing. Three tests indicate that the thermal-inactivation point of the virus lies between 64° and 68°C (Table 5). There appeared to be some loss of virus, however, even at 60°C, and there was a progressive reduction in active virus with rise in temperature until all virus was inactivated.

Longevity in vitro.—Juice extracted from diseased plants was placed in stoppered 50-cc flasks held at room temperature. At intervals, 2-cc quantities of juice were removed and used to inoculate test plants. There was considerable variation in results in the different tests, but the virus retained activity in all tests for at least 2 days, and in one test it retained activity for 7 days, though not for 14 days. The results of three tests, selected as representative, are presented in Table 5.

Tolerance of dilution.—Fresh juice of infected plants of *Silene gallica* was diluted with tap water and used to inoculate test plants. The infectivity of juice of *S. gallica* decreased rapidly with dilution, indicating that the virus probably cannot withstand a dilution much in excess of 1:2000 (Table 5).

Other properties.—Tests indicate that the virus does not survive drying in juice on watch crystals or in leaves of diseased plants. The virus retained activity with no apparent loss in seeds of *Lychnis divaricata* and *Silene noctiflora* stored at room temperature for 992 and 643 days, respectively (Table 6). In fact, per cent of seed transmission showed increases in seeds of *L. divaricata* over the first 569 days of the test.

TABLE 5.—*Properties of Lychnis ringspot virus as determined by local-lesion counts following inoculation of plants of Chenopodium album*

Thermal inactivation				Longevity *in vitro*				Tolerance of dilution			
Temperature (°C) for 10 minutes	Av. no. local lesions per leaf in trials:			Period aged at 20-24°C	Av. no. local lesions per leaf in trials:			Dilution	Av. no. local lesions per leaf in trials:		
	1	2	3		1	2	3		1	2	3
Check[a]	42.6	165.6	36.3	Check[a]	36.7	19.0	70.0	Check[a]	46.0	146.0	124.0
60	22.6	20.0	27.6	8 hours	4.3	27.0	6.9	1-10	33.7	92.0	114.7
62	12.3	32.0	3.3	1 day	4.7	9.7	9.6	1-100	1.6	19.0	15.3
64	3.0	28.3	1.0	2 days	5.7	12.7	4.5	1-500	1.0	2.3	1.3
66	0.0	0.0	3.3	4 days	0.0	9.3	2.0	1-1000	0.3	0.3	0.7
68	0.0	0.0	0.0	7 days	0.0	0.0	3.0	1-2000	0.0	0.0	0.0
70	0.0	0.0	0.0	14 days	0.0	0.0	0.0	1-5000	0.0	0.0	0.0

[a] Fresh undiluted juice from *Silene gallica*.

TABLE 6.—*Transmission of Lychnis ringspot virus through seeds of Lychnis divaricata and Silene noctiflora after different periods of storage*

Test species	Age of seeds from harvest[a] (days)	% germination[b]	No. seedlings infected	% seed transmission
L. divaricata	82	26	0	0.0
	179	73	16	21.9
	522	70	26	37.1
	569	67	38	58.2
	831	93	56	60.2
	992	21	11	52.4
S. noctiflora	18	66	15	22.7
	187	72	30	41.7
	482	77	27	35.1
	643	46	14	30.4

[a] Seeds of *Lychnis divaricata* and *Silene noctiflora* were harvested October 15, 1955, and September 29, 1957, respectively.
[b] 100 seeds in each test.

RELATION OF THE VIRUS TO HOST TISSUES.—Lychnis ringspot virus is unusual in its ability to multiply and produce visible disturbances in the parenchyma tissue of a wide range of host plants in the absence of systemic infection in a large proportion of the host species. Apparently, the virus not only has difficulty in passing from parenchyma into the phloem but also tends to be inactivated in the phloem after it has effected entrance.

In tobacco and sugar beet, the virus may be restricted to local lesions over long periods. Lesions usually do not become necrotic in leaves of these plants, and the virus is free to continue invasion from infected cells at the periphery of the lesion. Some lesions continued to enlarge more or less equally in all available directions until they were more than 3 cm in diameter. Tests of juice from chlorotic spots and from adjacent green tissue of inoculated leaves of Samsun tobacco, sugar beet, and New Zealand spinach indicate that the virus was present in the chlorotic tissue and absent from the green tissue. It appears, in these plants, that either the virus had great difficulty in moving from parenchyma into the phloem or that the phloem was an unfavorable medium for the virus.

An unfavorable reaction of phloem to the virus is indicated by the type of injury produced in *Chenopodium album* and *C. amaranticolor*. Circular local lesions were produced on inoculated leaves of plants of these species. Some lesions spread inward along main veins through the petioles into the stem, often killing stem tissue for a distance of 2–3 cm. The virus did not produce symptoms in the terminal growth, and inoculations with juice from tops of infected plants failed to indicate presence of the virus. Movement to the root system was also slow. In one test, in which leaves of *C. amaranticolor* had been inoculated, 10 plants were selected in which the virus had moved out of inoculated leaves and had produced lesions 1–3 cm long on the stems. The tops of these plants were removed about 1 cm below the lowest lesion. The plants later produced shoots from axillary buds that remained free of symptoms, indicating that the virus had not entered the root system. The sporadic but relatively fast movement of the virus out of some of the inoculated leaves suggests that the virus may be able to enter the phloem under certain conditions. Failure of the virus to invade the plant extensively indicates, however, that it usually is not able to move long distances in the phloem before it is inactivated.

Other results indicate that the virus may occasionally be able to enter the phloem of some species of plants and move considerable distances before it again enters the parenchyma. Chlorotic and necrotic lesions have appeared on plants of Samsun tobacco and petunia some distances from the points of inoculation although the intervening parts appeared normal. Tests of chlorotic and normal tissues from adjacent areas indicated that the virus was present in considerable concentrations in the chlorotic areas and absent from the green tissue. In addition, branches wholly free of symptoms were produced on some infected petunia plants that showed marked symptoms on other branches. Again, tests indicated that the virus was present only in the branches that showed symptoms. Some branches that at first were free of symptoms, later developed local chlorotic or necrotic spots on leaves or stems, indicating that the virus moved into these branches after they had remained free of infection in earlier periods of growth.

Results were somewhat similar in sugar beet plants. Some plants that showed lesions on the inoculated leaves later showed lesions on uninoculated leaves. In some instances, lesions continued to appear on successive leaves for several weeks in the absence of evidence of infection at the growing point. Usually, there was no evidence of infection of the growing point, and the plants eventually recovered, apparently with complete loss of the virus. In a few plants, the virus did move into the growing point, where it produced distorted, dwarfed leaves with rings and spots. It therefore seems probable that in sugar beet the virus is capable of moving sporadically into and out of phloem and of invading parenchyma in widely separated areas, often without reaching the growing plant. If the virus invaded the growing point, however, all growth produced thereafter was abnormal and there was little evidence of recovery.

So far as has been determined, the virus causes disturbances in all invaded tissue in tobacco, petunia, New Zealand spinach, and sugar beet. It therefore seems probable that, in these and perhaps in several other plants, distribution of virus may be indicated rather accurately by the presence or absence of symptoms.

PROBABLE RELATIONSHIP OF THE LYCHNIS RINGSPOT VIRUS TO OTHER VIRUSES.—The "ringspot viruses," placed by Holmes (4) in his genus *Annulus*, have certain rather distinctive characteristics in common. Induced symptoms are more or less similar, no insect vectors have been found, and infected plants often recover. The Lychnis ringspot virus seems to be related

to this group, but certain of its host plants show little evidence of recovery.

In host range and symptoms, the virus differs significantly from tobacco ringspot virus as described by Wingard (6) and Delphinium ringspot virus as described by Severin and Dickson (5).

Symptoms induced on aster in Florida by a ringspot virus described by Anderson (2) are similar to those induced on aster by the Lychnis ringspot virus. The thermal-inactivation point of the Florida virus, however, is about 10°C higher than that of the Lychnis ringspot virus, and the host range is different.

Harrison (3) recently described a ringspot virus of sugar beet in England, but this virus is soil-transmitted, does not always produce distinct symptoms on sugar beet, and invades tobacco and tomato systemically.

From the evidence available, it appears that the Lychnis ringspot virus has not been described previously; in fact, there is still no evidence as to the extent of its occurrence and distribution under natural conditions. The virus is therefore considered to be a previously undescribed virus belonging to the ringspot group of plant viruses.

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PARTIAL PURIFICATION AND ASSOCIATION OF FILAMENTOUS PARTICLES WITH THE YELLOW MOSAIC DISEASE OF BEAN[1]

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SUMMARY

Rate- and equilibrium-zonal density-gradient techniques were partially successful in the separation of bean yellow mosaic virus (bean virus 2) from normal plant constituents. Infectivity occurred in a definite region of the density-gradient tubes, although this region was not opalescent enough to be detectable visually. Electron micrographs of material from the infectious regions showed the virus particles to be elongated flexuous rods about 20 mμ wide and predominantly 790±40 mμ long.

BYMV (bean yellow mosaic virus; bean virus 2) has been regarded as probably being a long flexuous particle. De Bruyn Ouboter et al (5) published a picture of a single long filament that they thought might be the virus particle, and van der Want (8) also concluded that the virus was filamentous. Brandes and Quantz (3), using Johnson's exudate method (6), obtained particles 15 mμ wide and predominantly 750 mμ long from diseased plants, but not from healthy plants. The present note gives confirmatory evidence, gained by electron microscope and infectivity studies, that BYMV is indeed a flexuous rod. In addition, it shows that the difficulties encountered in purifying by standard methods can be largely overcome by using density-gradient techniques (1, 2). It leaves unsolved the problem of finding the high-yielding virus strains and/or hosts and environmental conditions necessary for producing visible virus zones.

MATERIALS AND METHODS.—The BYMV was originally isolated from red clover and cross-protected against a known strain.[2] The virus was grown in bean (*Phaseolus vulgaris* L. var Bountiful). Bean was used as the systemic infectivity assay plant throughout these studies since this BYMV isolate did not produce local lesions on *Crotalaria spectabilis* Roth (4). Infectivity assays, in which 0.01*M* Na_2SO_3 was always used as the diluent, were usually made at 2-4 dilutions between 10^{-1} and 10^{-3}, using an average of about 30 assay plants per dilution. Approximate comparisons of the relative active virus content of samples assayed at the same time were made by the preliminary estimate method of Brakke (2). Control plants were always included in the experiments.

All centrifugations, except preliminary low-speed runs at about 1500 rpm, were made on a Spinco Model L preparative ultracentrifuge. Low-speed runs were made at 15,000 rpm (maximum values given) for 10 minutes; high-speed runs were made at 29,000 rpm for 1 hour when the No. 30 rotor was used and 39,000 rpm for 40 minutes when the No. 40 rotor was used. Density-gradient rate and equilibrium-zonal centrifugations were performed at 24,000 rpm with an SW 25.1 rotor by the methods of Brakke (1, 2). The solvent used for the sucrose and plant samples was 0.01*M* Na_2SO_3. The tube temperatures reached about 18°C by the end of the rate runs.

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TABLE 1.—*Infectivities of preparations removed from density-gradient tubes after rate-zonal centrifugation* [a]

Amount of tissue[b] used to produce the extract for each tube	Distance from meniscus (mm)	Relative infectivities (%)
10	18–24	6
	25–35	100
	36–42	5
166	22–27	5
	28–33	100
	34–39	24
250	13–23	0
	27–37	100
	41–49	14

[a] Two-hour centrifugation at 24,000 rpm.
[b] Starting wet weight of tissue in grams.

TABLE 2.—*Infectivities of preparations removed from density-gradient tubes after equilibrium-zonal centrifugation* [a]

Centrifugation time (hr.)	Distance from[b] tube bottom (mm)	Relative infectivities (%)
4	25–40	0
	20–24	100
	16–19	37
5	22–26	4
	19–21	100
	16–18	39
6.5	20–23	3
	16–19	100
	14–16	84
7.5	20–23	2
	16–18	100
	14–16	94

[a] 24,000 rpm.
[b] Actual measurements were taken from the bottoms of the tubes because of evaporation that sometimes occurs during long periods of centrifugation. Approximate conversions to give readings from the meniscus can be obtained by subtracting the given distances from 61 mm.

RESULTS AND DISCUSSION.—*Preparation of material for rate-zonal centrifugation.*—Usually, systemically invaded trifoliate leaves of bean that had been infected 12-14 days at 80°F were used, since these leaves contained about four times as much virus per unit wet weight as did inoculated primary leaves. Small amounts of tissue were macerated in a mortar with a pestle, whereas larger samples were either minced with a meat grinder or homogenized in a Waring Blendor. Two volumes of 0.01*M* Na_2SO_3 were added to the plant tissue in these extractions. Previous tests measuring the infectivities of the supernatants and pellets of samples centrifuged 10 minutes at 15,000 rpm showed that the virus was less likely to aggregate in 0.01*M* or 0.1*M* Na_2SO_3 than in 1.0*M* Na_2SO_3, water, 0.1*M* K_2HPO_4 or 0.1*M* KH_3PO_4. A loss of one quarter to one third of virus could be expected by the low-speed centrifugation.

Initial clearing methods tested involved freezing, chloroform-butanol extraction (7), heating 10 minutes at 50°C and 40 minutes at 40°C at pH values of 6, 7, and 8 (2). All techniques resulted in a loss of infectivity, and none resulted in the appearance of a visible virus zone in the rate tubes, undoubtedly because the virus concentrations were not high enough to give light scattering greater than that occurring in the regions

Fig. 1. Bean yellow mosaic virus particles shadowed with uranium and photographed with a Siemens Elmiskop I (×42,500).

above and below the infectious region. In the experiments reported below, centrifugation was the preliminary clearing method employed. The extracted juice was first centrifuged 10 minutes at 1500 rpm. The supernatant was then further clarified in the Spinco, and this step was followed, depending on the amount of tissue involved, by one or two cycles of differential ultracentrifugation. The supernatant from the final low-speed run was subjected to rate-zonal centrifugation.

Rate-zonal centrifugation.—Regardless of the amount of tissue used, in all the nine runs assayed, little or no infectivity was found in the region between the meniscus and 25 mm below the meniscus. Most of the infectivity was found in the 26–36-mm region. The region below this usually gave little infectivity if low concentrations of clarified material (i.e., the material from 10 g of tissue in each tube) were used. When larger amounts of tissue were involved, the relative infectivity further down the tube increased (Table 1). Electron micrographs of samples removed from these infectious areas revealed many long flexuous particles mixed with amorphous material. The particles were not present in the noninfectious healthy plant samples taken from the same tube level although the amorphous material was present. These flexuous rods, which were invariably associated with the infectious unit when separated or concentrated by physical means, had a uniform width of about 20 mμ. It was difficult to measure their lengths accurately because most of the particles lay straight for only a small portion of their length. However, about 75% of them were certainly 790±40 mμ long, and another 15% were twice this length.

Equilibrium-zonal centrifugation.—Five-ml samples from the most infectious region of the rate-zonal tubes were used for equilibrium-zonal centrifugation. At least 6.5 hours were required to reach equilibrium (Table 2). A green layer was noticeable in both control and virus samples, but below this region for about 2 mm, a very faint opalescent zone was sometimes seen in the virus tubes that was not present in the control tubes. These two regions were noninfectious in the control tubes, but they carried nearly all the infectivity, in approximately equal amounts after prolonged centrifugation, in the virus tubes. Electron micrographs taken of these two lower regions showed that flexuous rods and much non-uniform amorphous material were associated with the green layer, whereas the lowest region contained flexuous rods with very little amorphous material (Fig. 1). When the infectious material was removed, concentrated, clarified, and recycled on a rate-zonal tube, no opalescent zone was visible.

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STUDIES ON RESISTANCE IN TOMATO TO ROOT-KNOT NEMATODES AND ON THE OCCURRENCE OF PATHOGENIC BIOTYPES[1]

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SUMMARY

The factors for resistance or susceptibility to the root-knot nematode *Meloidogyne incognita* in tomato were found to be located in both the tops and roots of plants. They were not translocated across a graft union.

Larvae of root-knot nematodes penetrated the roots of resistant plants as freely and as rapidly as they penetrated roots of susceptible plants. However, the invasion of resistant tissues resulted in the death of tissues around the invading larvae and the subsequent death of the larvae. The effects on the cells surrounding the larvae were noted as early as 24 hours after root tips were inoculated and within 48 hours the cells appeared to be dead. Nematode larvae were dead within 96 hours after inoculation. Offspring of females which had developed in resistant plants formed galls on both resistant and susceptible plants which were indistinguishable from the galls formed by the parent populations on susceptible plants.

When larvae from *M. incognita incognita*, *M. incognita acrita*, and *M. arenaria arenaria* females which had matured on resistant Hawaii 5229 tomato plants were transferred generation after generation to other resistant plants new populations of each of the three subspecies virulent on Hawaii 5229 were developed. These parasitized resistant Hawaii 5229 tomato plants as severely as the parent populations parasitized susceptible STEP 174 tomato plants.

No variations in virulence to resistant or susceptible tomato plants were found in single larva cultures from the new population of *M. incognita incognita*. However, a few offspring of all cultures developed from single larvae of the parent populations parasitized and developed into mature nematodes on resistant plants.

Resistant varieties of several crop plants other than tomato were inoculated with the new virulent strain of *M. incognita acrita*. In general they were found to be resistant to the new as well as to the parent populations. Peanuts were not affected by either the parental or the new populations of *M. incognita acrita* or *M. arenaria arenaria*. Sweet potatoes were slightly to moderately galled by the new strain of *M. arenaria arenaria*, unaffected by the parent population of *M. arenaria arenaria*, and severely galled by both populations of *M. incognita acrita*.

There were some indications of morphological differences between the perineal patterns of the new strains and those of the parent populations. However, these differences were not great enough to distinguish between them by this character. The larvae of each of the populations were concluded to be new pathogenic strains of *M. incognita acrita*, *M. incognita incognita*, and *M. arenaria arenaria*.

Steiner (21) defined resistance to nematodes as the ability of the roots to resist penetration by this pest. Barrons (3), however, found that roots of resistant plants were penetrated by root-knot nematode larvae as freely as roots of susceptible plants. Christie (5) found that some roots were not penetrated by root-knot nematode larvae while others were. He stated that necrosis occurred when the roots of some resistant plants were penetrated. Mizogami (16) described necrosis in the invaded roots of resistant sweet potato, and this was confirmed by Shibuya (19) and by Dean and Struble (6). The latter also reported necrosis in resistant tomato roots invaded by *Meloidogyne* larvae. Liao and Dunlap (13) reported similar results with tomato except that larvae were arrested half embedded in the tissues.

The source of resistance used in this study originated from a cross by Smith (20) between *Lycopersicon peruvianum* L. and *L. esculentum* Mill. The progeny of the cross were backcrossed by Watts to *L. esculentum* (22). Through the continued efforts of several investigators this resistance has been transferred to *L. esculentum* (7, 8). Breeding lines currently in use carry resistance to *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, *M. incognita acrita* Chitwood, 1949, *M. arenaria* (Neal, 1889) Chitwood, 1949, and *M. javanica* (Treub, 1885) Chitwood, 1949. The nature and mechanism of this resistance is unknown. Barham and Sasser (1) found one of the lines from Hawaii to be resistant to 4 *Meloidogyne* spp. Barham and Winstead (2) found that resistance to all 4 species was controlled by a single, incompletely dominant gene, *Mi* (9).

Physiologic specialization in root-knot nematodes has been observed by many workers (4, 5, 15, 18).

This study was undertaken to determine the nature of resistance and to elucidate some related factors. Included were studies on: 1) location of resistance; 2) penetration and subsequent development of nematodes within resistant roots; and 3) development of strains of the nematodes.

MATERIALS AND METHODS.—Used in the study were two tomato breeding lines—one root-knot susceptible,

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STEP (Southern Tomato Exchange Program) 174[3]; and one resistant, Hawaii 5229[4].

Plants for use in most greenhouse studies were grown to transplant size in sand in flats and watered with a nutrient solution described by Hoagland and Arnon (11). Plants in the 2–4 true-leaf stage were transplanted to a potting mixture consisting of 5 parts soil to 2 or 3 parts sand. The soil was fumigated with methyl bromide at the rate of 3 lb. per 100 sq. ft. of soil. Six-inch pots were used in most greenhouse tests.

Root-knot nematodes used in these studies were *M. incognita incognita*, *M. incognita acrita*, and *M. arenaria arenaria*. Inoculations were usually made with larvae. These were obtained by placing egg masses in water and allowing the eggs to hatch. The larvae were picked out, counted, and placed in vials at room temperature for use in inoculations. Inoculated plants were maintained at a greenhouse temperature of 25–32°C. Root-knot examinations were usually made 3 months after inoculation. The soil was washed from the roots in running water. Disease ratings were based on the amount of galling and on egg mass production: 0, no galls and no egg masses; 1, few galls and no egg masses; 2, moderate galling and a few egg masses; 3, moderate to severe galling and numerous egg masses; 4, very severe galling and large, numerous egg masses.

Penetration of STEP 174 and Hawaii 5229 by *M. incognita incognita* larvae was studied by examining material killed and stained at various intervals after inoculation. The acid-fuchsin–lactophenol technique described by Goodey (10) was used. For histological studies the roots were changed from lactophenol to distilled water, and then dehydrated with dioxane as described by Goodey (10). The procedure outlined by Sass (17) for infiltration, embedding, sectioning, mounting, and staining (safranin-fast green) was used.

In the studies on the development of nematodes in resistant plants the roots were killed and fixed in Craf III reagent (17). The roots were then placed in 100 ml of 10% ethanol containing 2 ml of a 1% solution of acid fuchsin (12). They were dehydrated and cleared by a short ethanol-xylol series.

Again the procedure outlined by Sass (17) was used for infiltration, embedding, mounting, and staining. The safranin-fast green stain was used. Other materials and methods related to specific experiments will be indicated in connection with the description of the experiments concerned.

EXPERIMENTAL RESULTS.—*Location of resistance.*—Whether the factors for resistance or susceptibility were localized within the roots or present throughout the plant, was investigated. Reciprocal approach grafts between resistant and susceptible tomato lines were used. There was a period of 2 weeks between the removal of the unwanted stock and scion and the inoculations with larvae of *M. incognita incognita*. Root inoculations were made by placing about equal quantities of infested soil into each of the pots. Leaves were inoculated with larvae by a method described by Linford (14). Four types of plants were tested: 1) resistant plants; 2) resistant stocks with susceptible scions; 3) susceptible stocks with resistant scions; and 4) susceptible plants. Inoculated plants were kept in the greenhouse at about 30°C.

The roots of 8 plants of each of the 4 types were inoculated. After 8 weeks the roots of resistant plants and of resistant stocks with susceptible scions had very few or no galls. Conversely, roots of susceptible plants and susceptible stocks with resistant scions showed severe gallings.

Leaves of each of the 4 plant types were inoculated in 2 tests. In the first test, 10 each of susceptible and resistant plants and 6 each of the grafted plants were inoculated. In the second test, 10 and 5 plants, respectively, were inoculated. No leaf galls formed in either test on resistant plants or on resistant scions on susceptible stocks. Of the 10 susceptible plants in each test, 7 had galls in the first test, and 8 had galls in the second test. Of the susceptible scions on resistant stocks, 4 of 6 had galls in the first test, and 4 of 5 had galls in the second test.

Penetration studies.—Studies were undertaken to determine whether larvae of *M. incognita* penetrated Hawaii 5229 roots as rapidly and as abundantly as they invaded STEP 174 roots. Seeds were disinfested by placing them on a dry filter paper in a Petri dish containing a second filter paper to which 2 drops of Panogen (cyano (methylmercuri) guanidine), had been added. After 24 hours the seeds were removed to other Petri dishes containing moist filter papers and were germinated at 24°C. When the primary roots were 1–2 in. long, 2 seedlings were placed in each of several Petri dishes containing moist filter papers. The root tips were covered with fine sterilized soil (150-mesh), and 100 nematode larvae in suspension were syringed onto the soil around each root tip. The seedlings were maintained at 24°C. Roots were removed at 24, 48, 96, and 144 hours and were stained in hot acid-fuchsin–lactophenol and destained in cold lac-

TABLE 1. *Average number of M. incognita incognita larvae penetrating resistant Hawaii 5229 and susceptible STEP 174 tomato seedlings*

Plant	Test	Average number[a] of larvae per root at 24 hours	48 hours	96 hours	144 hours
Hawaii 5229	1	3.9	2.9	9.4	7.1
STEP 174	1	2.6	7.5	14.0	12.1
Hawaii 5229	2	7.4	14.7	33.5	39.7
STEP 174	2	4.3	16.3	41.4	44.9

[a] The numbers of larvae that entered the roots of the two tomato lines were not significantly different. Each figure represents an average of 10 roots in test 1, and 15 roots in test 2.

[3] STEP 174 was developed by C. F. Andrus, of the Southeastern Vegetable Breeding Laboratory, Charleston, South Carolina.

[4] Hawaii 5229 was developed by J. C. Gilbert and D. C. McGuire at the Hawaii Agricultural Experiment Station, Honolulu, Hawaii.

tophenol. Counts of larvae found inside or partially inside the roots are presented in Table 1. There were no significant differences between the number of larvae that entered roots of susceptible plants at a given time.

Roots of both hosts were penetrated within 25 hours of inoculation. The number of larvae penetrating increased with time up to 144 hours. In 2 inoculation experiments the percentage of larvae penetrating was larger in the second than in the first. The difference found was probably due to the method of examination: In the first inoculation the roots were retained intact

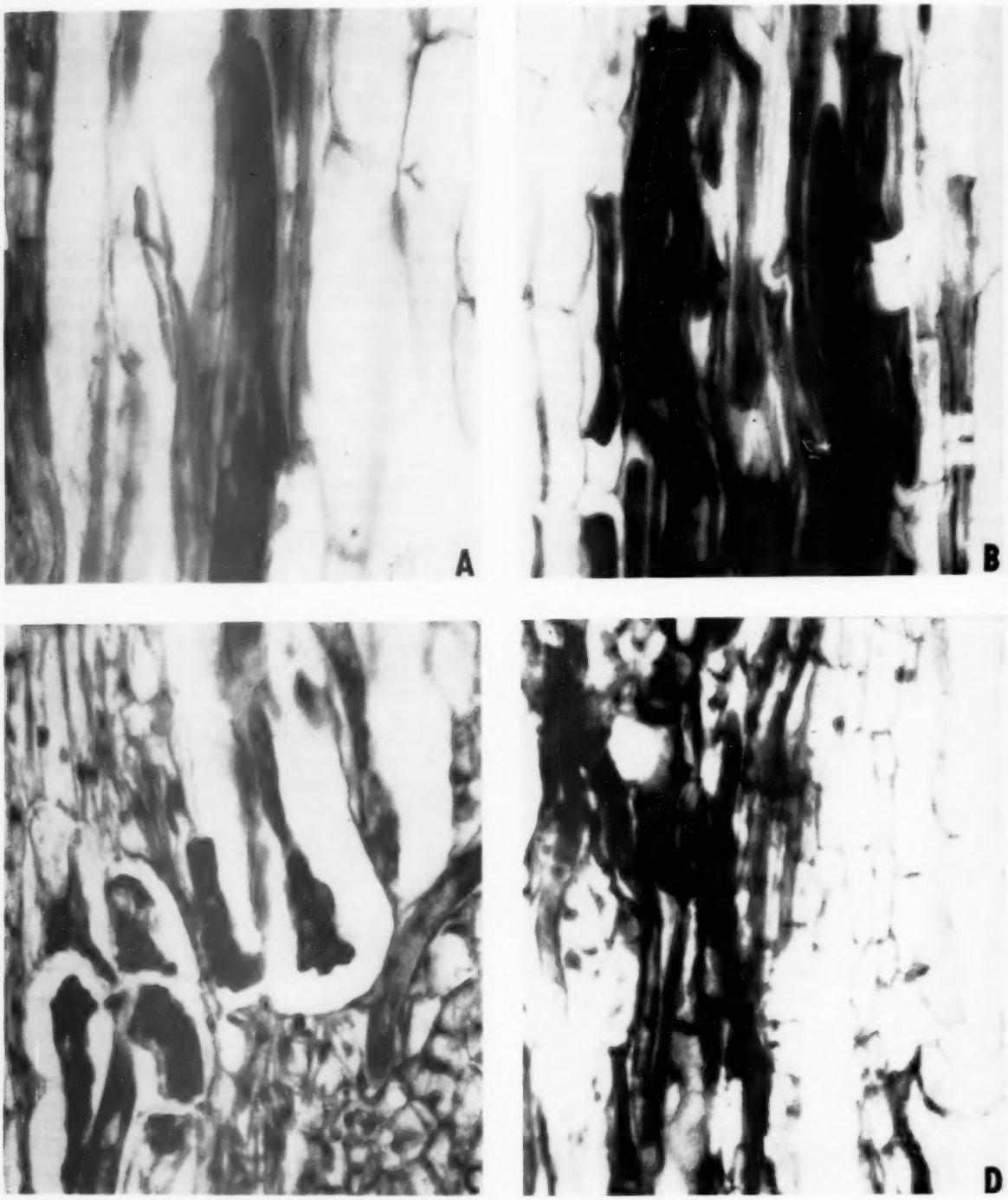

Fig. 1. The reaction of STEP 174 (A,C) and Hawaii 5229 (B,D) tomato roots to invasion by *M. incognita incognita* larvae. **A,B)** 24 hours after inoculation. **C,D)** 144 hours after inoculation.

(for histological studies), examined *in toto*, and only nematodes clearly seen were counted; in the second test the roots were dissected to make counts more complete. Observations of live unstained root rips revealed that the areas around the nematodes in resistant roots were necrotic. The cells and their contents were brownish, and the protoplasm was coagulated into a mass in the center of each cell. Therefore, histological studies were made of these tissues. Portions of the roots containing nematodes were embedded in tissuemat, sectioned, and stained with safranin and fast green. The tissues surrounding the nematodes in resistant roots were very deeply stained with safranin. At the 24-hour incubation period the deep red areas were mostly around the anterior end of the larvae (Fig. 1-B). As the incubation period increased, the extent of the deeply-stained areas increased. No deep red areas were observed around larvae in roots of susceptible plants at any of the intervals (Fig. 1-A). Giant cells could not be found in any of the resistant roots (Fig. 1-D), but were easily discernible in susceptible roots from the 96- and 144-hour intervals (Fig. 1-C).

Larvae were teased from live roots at various intervals to ascertain their viability. Larvae taken from resistant roots 48 hours after inoculation were motile (hence, alive), and when punctured with a needle their body contents gushed out. However, larvae removed from roots 96 hours after inoculation were non-motile and apparently dead. Their body broke into 2 pieces when punctured, and no contents gushed out.

Development of nematodes in resistant plants.—Limited reproduction of each of the 3 *Meloidogyne* subspp. was known to occur on resistant plants. The galls that were produced resembled those on susceptible plants. Histological comparisons were made between the internal structures of the two. Seedlings in sand in 3-in. clay pots were inoculated with 200 *M. incognita incognita* larvae per pot. The plants were removed at intervals, one plant of each line at each interval, and the roots were killed and fixed.

Giant cell formation was considerable in roots of Hawaii 5229 plants 19 days after inoculation. Female nematodes were apparently mature, but no egg masses were present. Galls on STEP 174 roots of comparable age were similar, but egg sacs were forming. At 26 days, egg sacs were forming on females in Hawaii 5229 roots. Egg sacs in STEP 174 roots contained many eggs, some with fully formed first- or second-stage larvae in them. This test was subsequently repeated, with the parental population maintained on STEP 174 plants and a population of *M. incognita incognita* that had been maintained for 15 months on Hawaii 5229 tomato plants. STEP 174 was inoculated with the parental population, and both STEP 174 and Hawaii 5229 were inoculated with the population from Hawaii 5229. Female development and egg production with both populations and on both crops were similar to that previously described for the parental population on STEP 174 plants. The new populations maintained for 15 months on Hawaii 5229 plants developed as rapidly on either host as did the parental population on STEP 174.

Culture of nematodes on resistant plants.—The 3 *Meloidogyne* subspp. to which Hawaii 5229 is resistant were known to produce on this tomato breeding line to a very limited extent. The virulence of progeny from nematodes that developed on resistant plants was therefore compared with the virulence of the original populations. Only *M. incognita incognita*, *M. incognita acrita*, and *M. arenaria arenaria* were included in these studies.

Hawaii 5229 plants were transplanted into nematode-infested soil. At 3 months, the roots were examined for root-knot severity and the roots and soil were used to inoculate other Hawaii 5229 plants. In subsequent inoculations, egg masses were collected and larvae were allowed to hatch. Plants were inoculated with about 200 larvae each and were left for 3 months before root-knot indices were obtained. All tests were replicated 4 times.

Very few galls were found on the plants after initial inoculation. However, galls became more numerous with each successive transfer from Hawaii 5229 to Hawaii 5229. The root-knot indices in the initial inoculation were: *M. incognita incognita*, 1.0; *M. incognita acrita*, 2.0; *M. arenaria arenaria*, 1.5. After the third transfer the respective indices were 3.8, 3.5, and 3.0. Hawaii 5229 had little resistance to these latter populations. The population built up and maintained on Hawaii 5229 plants were designated as B populations, and the original populations maintained on susceptible plants were designated as A populations.

The virulence of the A and B populations was compared on Hawaii 5229 and STEP 174, 16 plants each. Plants of each type were inoculated with larvae of each of the 6 populations. Results of this test are presented in Table 2, and the types of reactions obtained are illustrated in Fig. 2. Population A of each subspecies was less virulent on Hawaii 5229 than was the B population. *M. incognita acrita* A and *M. arenaria arenaria* A were more virulent on Hawaii 5229 than *M. incognita incognita* A. The B populations were as virulent on Hawaii 5229 as on STEP 174. On STEP 174 the A populations of *M. incognita incognita* and

TABLE 2.—*Root-knot index of 2 tomato lines inoculated with populations A[a] and B[b] of 3 subspecies of root-knot nematodes*

	M. incognita incognita		M. incognita acrita		M. arenaria arenaria	
Plant	A	B	A	B	A	B
Hawaii 5229	0.06[c]	3.69	1.13	3.88	1.94	3.63
Mean difference	3.63**		2.75**		1.69**	
STEP 174	4.00	3.50	3.94	3.69	3.69	3.69
Mean difference	0.50*		0.25*		0.00	

[a] This represents the population to which the Hawaii 5229 line is normally resistant.

[b] This represents the population which has been built up on the Hawaii 5229 line.

[c] Each figure represents an average of 16 plants.

* Significant at the 5% level.

** Significant at the 1% level.

M. incognita acrita were significantly more virulent (at the 5% level) than the B populations as determined by the *t* test of significance. In subsequent tests, B populations maintained continuously on Hawaii 5229 plants were as virulent as the A populations on STEP 174.

Tests were made to determine the virulence of the B populations on susceptible varieties of other crop plants, and to determine if this factor would be altered by maintenance on susceptible plants. Dixie Bright

Fig. 2. Representative reaction of: **A)** Hawaii 5229 tomato roots inoculated with larvae of *M. incognita incognita* B, *M. incognita acrita* B, or *M. arenaria arenaria* B that developed on Hawaii 5229 roots; **B)** STEP 174 tomato roots inoculated with either the A or B population of the 3 *Meloidogyne* subspp. tested; and **C)** Hawaii 5229 tomato roots inoculated with the A population of the 3 *Meloidogyne* subspp. tested.

101 tobacco, *Nicotiana tabacum* L.; Perkin's Mammoth Long Pod okra, *Hibiscus esculentus* L.; Model cucumber, *Cucumis sativa* L.; and STEP 174 tomato were inoculated with each of the B populations. Root-knot indices were determined 3 months after inoculation. For example, plants of Hawaii 5229 and STEP 174 tomatoes, and tobacco were inoculated with larvae taken from egg masses formed on tobacco. Similar inoculations were made with inoculum produced on okra, cucumber, and STEP 174 tomatoes. Each population was replicated 5 times on each crop. Larvae were transferred at 3-month intervals. Virulence to Hawaii 5229 tomatoes was not decreased when any of the B populations were maintained 3, 6, or 9 months (1, 2, or 3 transfers) on susceptible plants. Population B of *M. incognita incognita* was the least virulent of the B populations on okra; however, all 3 incited severe root-knot.

Resistant varieties of other crop plants were inoculated with *M. incognita acrita* B. Plants were grown in 4-in. clay pots, and 500 larvae were placed in each pot. Varieties tested were Coker 139 tobacco; NC-2 peanuts, *Arachis hypogea* L.; Fulgrain oats, *Avena sativa* L.; Laredo soybeans, *Glycine max* (L.) Merr.; Rowan lespedeza, *Lespedeza stipulacea* Maxim; "Goatweed" pepper, *Capsicum* sp. (line 405B from W. W. Hare, Mississippi State College); crotalaria, *Crotalaria striata* D. C.; Alabama No. 1 pole snapbean, *Phaseolus vulgaris* L.; Iron cowpea, *Vigna sinensis* (L.) Endl.; and Massey strawberry, a *Fragaria* sp. A few galls and a moderate number of mature females with egg masses were found on cowpea. In a separate experiment, 2 lines of Iron cowpeas were inoculated with larvae of the A and B populations. Both nematode populations reproduced moderately on each line. No galls or females were found on any of the other resistant plants tested.

Single-larva inoculations.—Since the B populations were derived from the A populations, it was probable that variation for pathogenicity occurred within the original populations. A study was made to ascertain this probability. Single-larva inoculations were used in an attempt to select populations that differed in pathogenicity.

Seeds of Hawaii 5229 and STEP 174 tomatoes were germinated in Petri dishes. Single seedlings were transferred to other Petri dishes containing moist filter papers. The tip of each root was covered with sand. Five egg masses each of the A and B populations of *M. incognita incognita* were placed in B.P.I. watch glasses, one egg mass per watch glass. After the eggs hatched, individual larvae were picked out with a micropipette and one larva was placed in the sand around each primary root tip. The pipette was rinsed after each larva was ejected. Ten plants each of Hawaii 5229 and STEP 174 tomatoes were inoculated from each egg mass of the B population. Ten plants of the STEP 174 line were inoculated from each egg mass of the A population. After 48 hours at room temperature the seedlings were transplanted into pots in the greenhouse maintained at about 24°C. When galling was observed the roots were washed free of soil, and egg masses were picked from galled roots for use in further studies. Nine Hawaii 5229 plants and 3 STEP 174 plants became infected by individual larvae of the B population.

Larvae from infected plants were used to inoculate other Hawaii 5229 and STEP 174 plants. Five plants of each tomato line were inoculated with 500 larvae from each infected plant. At 2 months, all Hawaii 5229 plants inoculated with larvae of population B, whether derived from Hawaii 5229 or STEP 174 plants, had root-knot indices of 3-4. Hawaii 5229 plants inoculated with larvae of the A population collected from STEP 174 rated 0–1. All 9 A populations reproduced to some extent on Hawaii 5229. The disease indices of these populations on STEP 174 plants were 3–4.

Morphological studies with nematodes of the B populations.—Several perineal patterns of each species were examined and identified. In B populations this character conformed morphologically within the range of variability for the species. Few patterns of any of the B populations, however, conformed closely to the species type. Almost all of the patterns had more arch than is characteristic of *M. arenaria arenaria*. These arches were not as high as those characteristic of *M. incognita incognita* and *M. incognita acrita*. Confirmations were made by Dr. J. N. Sasser, North Carolina State College.

Since all the patterns were very similar, a host study was made with *M. incognita acrita* and *M. arenaria arenaria*. The hosts used included sweet potato, which is susceptible to the former and resistant to the latter, and peanut, which has the reciprocal reaction. These hosts were inoculated with both populations of *M. incognita acrita* and *M. arenaria arenaria*. Eight weeks after inoculation, peanuts were not galled by either the A or B populations of the 2 subspecies. Sweet potatoes were severely galled by both populations of *M. incognita acrita*, slightly galled by *M. arenaria arenaria* B, and not galled by *M. arenaria arenaria* A.

Measurements were made of larval lengths. Egg masses of both populations of *M. incognita incognita*, *M. arenaria arenaria*, and *M. incognita acrita* were collected from tomato roots, placed in water and held at room temperature. At 48 hours and 14 days, 25 larvae of each were picked out, placed on a slide, and killed by gently heating. A camera lucida sketch was made of each larva, and larval lengths were calculated from these drawings (Table 3).

In all cases, larvae of the A populations were significantly longer than those of the B populations. Larval lengths of *M. incognita incognita* A were not different from those of *M. incognita acrita* A, but both were significantly shorter than *M. arenaria arenaria* A larvae. Larval lengths of the 3 B populations were not different.

In a subsequent test, *M. incognita incognita* B larvae were harvested from STEP 174 and Hawaii 5229 plants, and *M. incognita incognita* A larvae from STEP 174 plants. Larval lengths were compared at

TABLE 3.—*Length (μ) of Meloidogyne larvae taken from 2 populations within each subspecies as indicated*

	M. incognita incognita		M. incognita acrita		M. arenaria arenaria	
	A[a]	B[a]	A	B	A	B
Range	315–480	329–425	343–433	295–418	364–535	299–453
Average of 50 larvae	388	364	388	374	461	369
LSD (0.01) between A & B populations	13		11		19	
LSD (0.01) between means = 17						

[a] Population A indicates the population to which Hawaii 5229 tomatoes are resistant; population B indicates the population to which Hawaii 5229 tomatoes are susceptible.

4 days and 14 days after the egg masses were harvested (Table 4). Individuals of the B population were significantly longer on STEP 174 than on Hawaii 5229. On STEP 174, larvae from population A were longer than larvae from population B.

Inoculations for the data presented in Table 3 were made in early December, 1957, and measurements were made the following March. Inoculations were made in May, 1958, and the data given in Table 4 were collected the following August. In the first test, samples of both the A and B populations of *M. incognita incognita* and *M. incognita acrita* larvae taken 2 days after egg masses were collected were significantly longer than larvae taken 12 days later. This was also true for *M. arenaria arenaria* B; however, *M. arenaria arenaria* A larvae were longer after the 14-day interval than those measured 12 days earlier. The larvae were taken at random in each case; therefore the order of hatching and the time a larva spent in the water were not known. Hence, no correlation can be drawn from these data between age and larval length or between sequence of hatching and larval length. These results suggest that environment and host affected larval length appreciably.

DISCUSSION.—Root and leaf reactions of resistant, susceptible, and reciprocally grafted plants to *Meloidogyne* spp. indicated that the factors for resistance or susceptibility were inherent within individual cells. The level of resistance in roots and leaves was not altered by the presence of susceptible tops or roots, or vice versa. This also indicated that the factors for resistance or susceptibility either are not translocated or do not cross graft unions.

Penetration of resistant roots by root-knot nematode larvae has been reported by several investigators (3, 5, 6, 7, 8, 16, 19). Hawaii 5229 tomato roots were penetrated as freely and as rapidly as were STEP 174 roots. Hence, resistance in Hawaii 5229 is not due to mechanical barriers to penetration. It does not appear probable that a chemical is present in resistant tomato roots that arrests the nematodes before they have completely penetrated, as suggested by Liao and Dunlap (13) in their studies on resistance in *L. peruvianum*. Nematodes that had gained only a partial entrance were observed in both Hawaii 5229 and STEP 174 roots in aproximately equal numbers.

Various substances such as suberin, lignin, and necrotic tissues have a high affinity for safranin. The invaded roots of resistant Hawaii 5229 plants were deeply stained by safranin whereas those of susceptible STEP 174 plants did not take the stain. When inoculated fresh roots were torn apart, the tissues around the nematode were observed to be necrotic. This hypersensitive reaction was evident as early as 24 hours after inoculation. Dean and Struble (6) found that necrosis in resistant sweet potatoes appeared within 48 hours.

Previous writers (3, 5, 6, 13, 16, 19) postulated that larvae that penetrated resistant roots died of starvation. In these studies, larvae appeared to be dead within 96 hours. Larvae left in water at room temperature remained active for 3 or more weeks. This difference may have been due to an increased expenditure of energy by the larvae during penetration. If this occurred and the larvae could obtain no nourishment, they possibly could have starved in the shorter period. However, it seems more probable that the death of root tissues may have generated and released substances that were toxic to the nematodes and quickly brought about their death.

A few larvae occasionally develop to maturity in roots of resistant plants. Furthermore, progeny of nematodes that developed on Hawaii 5229 plants (the B populations) did not incite the hypersensitive reaction. Therefore, the reacting substance was apparently not specific for the presence of the nematode, but only to larvae of the A populations. This suggests that most larvae of the A populations excrete some substance that reacts with substances within the plant, resulting in tissue necrosis. Shibuya (19) suggested that the difference between resistant and susceptible sweet potatoes would be found in the protein fraction. This may be true for tomatoes also. Nematode larvae of the A populations may secrete a substance that coagulates the proteins of resistant plants, but not of susceptible plants. In such a reaction, some by-product toxic to the nematode might be formed and

TABLE 4.—*Length (μ) of M. incognita incognita larvae of the A and B populations*[a]

	Population B		Population A
	Hawaii 5229	STEP 174	STEP 174
Range	278-429	297-446	344-518
Average of 100 larvae	365	379	402
LSD (1%) between the B populations maintained on Hawaii 5229 and STEP 174 and between A and B populations maintained on STEP 174 = 10			

[a] Population A indicates the population to which Hawaii 5229 tomato plants are resistant; population B refers to the population to which Hawaii 5229 plants are susceptible.

would explain the early death of the larvae. The protein system has been used as an example and may not be the affected system; nevertheless, the over-all reaction is lethal to the cells surrounding the nematodes and to the invading nematodes. These studies did not elucidate the nature of the physiological or biochemical factors associated with resistance; however, they indicated the high degree of specificity involved.

Physiologic races of root-knot nematodes have been observed previously (4, 5, 15, 18), but no reference to the selection of new strains from greenhouse cultures was found in the literature. A few larvae of the 3 subspecies tested developed and reproduced on Hawaii 5229 tomatoes. These were built up within 1 year into populations that readily parasitized Hawaii 5229 tomatoes. These populations are physiologically distinct from the parent populations and are considered to be new strains. These new strains were just as virulent on susceptible tobacco, okra, cucumber, and tomato as were the parent populations. However, neither the A or B population of the peanut root-knot nematode, *M. arenaria arenaria*, was pathogenic on peanut. Maintenance of the new strains on these susceptible plants for 3, 6, or 9 months did not affect their virulence to Hawaii 5229. Therefore, the change to new strain was not caused by the resistant plants, but the plants served merely to screen out the individuals with the inherent genetic ability to develop in the presence of the *Mi* gene. The new strain or population should be considered genetically stable. The specialized nature of these new strains is indicated by their inability to parasitize resistant varieties of other crop plants. This also indicated the specialized nature of the factors controlling resistance. Strains of root-knot nematodes have recently been observed in the field in Hawaii and Australia (personal communications with Dr. J. C. Gilbert, University of Hawaii, Honolulu, and Dr. R. D. Brock, Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra, Australia) that attack tomato plants carrying the *Mi* gene for resistance, as in Hawaii 5229.

Perineal patterns of each of the 3 new strains differed from those of the parent populations. The differences were not sufficient to exclude any of the new strains from the parental type. The differences fell within the range of previously observed natural variation within each subspecies, but were more consistent. The patterns found in each of the 3 new strains were more similar to each other than to the parental types.

The larvae of the new strains were significantly shorter than those of the parent populations. There were no significant differences among the 3 new strains in lengths of the larvae. This, along with the similarity of perineal patterns, suggests that the 3 new strains may be more closely related to each other than each to its parent population. *M. incognita acrita* B more nearly fitted its species characteristics than did *M. incognita incognita* B or *M. arenaria arenaria* B.

A few mature females with egg masses developed on the roots of tomato plants carrying the *Mi* gene for resistance with each of the 9 single-larva cultures of *M. incognita incognita* used in these studies. Furthermore, all populations of *M. incognita incognita* A, *M. incognita acrita* A, *M. arenaria arenaria* A, and *M. javanica* A tested by the authors on tomato plants carrying the *Mi* gene for resistance have reproduced to a limited extent. It appears that the potential genetic makeup that enables *Meloidogyne* sp. A to develop into populations of *Meloidogyne* sp. B is widespread. Other workers (1, 2, 8, 9) have also noted the formation of a few females with egg masses.

A report of variation in the genus *Meloidogyne* in pathogenicity, virulence, morphology, and other characters is neither new nor unique. In this report, however, the variation in pathogenicity is specific for a particular gene *Mi* found in tomato. Furthermore, this specificity was associated with certain morphological characters, and all were found within 3 subspecies. As an increasing number of variants is found within and among the various species of *Meloidogyne*, the need for more precise work on variation and for better understanding of the basic mechanisms of variation becomes increasingly evident.

PLANT PATHOLOGY
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FACTORS AFFECTING SPORE GERMINATION OF TWELVE SPECIES OF TILLETIA FROM CEREALS AND GRASSES [1]

J. P. Meiners and J. T. Waldher [2]

SUMMARY

Light and an unknown soil factor, in addition to low temperatures, are necessary for maximum spore germination of *Tilletia contraversa*. However, the degree to which spores depend on light for germination varies among accessions and within an accession, depending on the year in which the spores were collected. Soil-extract agar proved equal to soil as a substratum for spore germination. Spores of species of *Tilletia* occurring on grasses were incubated at 5, 10, and 15°C. Optimum temperature for germination was 5° for *T. asperifolioides*, *T. bromi-tectorum*, *T. caries*, *T. contraversa*, *T. elymi*, *T. fusca*, *T. guyotiana*, *T. holci* and *T. scrobiculata*; 5° or 10° for *T. cerebrina*; 10° for *T. asperifolia*; and 15° for *T. pallida*. Studies with many collections of *T. bromi-tectorum*, *T. fusca*, and *T. guyotiana* revealed that optimum germination temperature was 5° for all collections, but rate of germination varied among species and collections of the same species. Light stimulated germination of spores of *T. fusca*, *T. guyotiana*, *T. holci*, *T. caries*, and *T. cerebrina* (1 collection), had no effect on *T. asperifolia*, *T. bromi-tectorum*, and *T. elymi*, and suppressed germination of *T. scrobiculata* and *T. cerebrina* (2 collections).

Introduction.—Knowledge of the factors that influence spore germination is a prerequisite to successful studies of the biology of *Tilletia* spp. The characteristics of spore germination are an important adjunct to a study of spore morphology and host symptoms in identifying members of this genus. Also, studies of the life history, cytology, genetics, and host range are greatly facilitated by a knowledge of factors essential for good spore germination. Such information is lacking for a majority of *Tilletia* spp., particularly those that occur on grasses. Only with species that occur on wheat (*T. caries* [DC.] Tul., *T. foetida* [Wallr.] Liro, *T. contraversa* Kuehn) have the factors that influence spore germination been extensively investigated. This paper reports studies of factors that influence spore germination in several species of *Tilletia* common on cereals and grasses in the Pacific Northwest.

Review of literature.—Of the species of *Tilletia* included in the investigations, a comprehensive study of factors affecting germination had been made only for *T. contraversa*. With this species it had been found that low temperature (9) and light (1, 2, 5, 12) are essential to satisfactory spore germination. Unknown soil factors also appeared to be necessary for maximum spore germination (2, 5).

Some of the results of the limited studies of spore germination in *Tilletia* species appear to be contradictory. Germination of a few spores of *Tilletia fusca* Ell. & Ev. was obtained by McAlpine (10) after 17 days' incubation at room temperature in a drop of boiled water. Fischer and Hirschhorn (4) germinated spores of this species as well as those of *T. asperifolia* Ell. & Ev., *T. cerebrina* Ell. & Ev., *T. elymi* Diet. & Holw., and *T. guyotiana* Hariot by soaking them at 2–3°C for several weeks and then placing them on water agar at room temperature. Holton and Siang (7) found that spores of *T. asperifolia* on 2% water agar germinated in 1 week at 15–18°, and in 2 weeks at 8°. Holton (6) found that lower temperatures were required for *T. elymi*, the spores germinating after incubating about 1 month at 5°C in the refrigerator. Likewise, Siang (13, 14) obtained good germination

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of spores of *T. cerebrina*, *T. fusca*, *T. guyotiana*, and *T. holci* (West.) DeToni at 5°C. Das (3) germinated spores of *T. holci* by subjecting them to pretreatment in 10% bleaching powder and then incubating 5–7 days at 1–3° in the presence of light, and subsequently for about 2 days at 18–20°.

MATERIAL AND METHODS.—Spore material of 12 species of *Tilletia* was obtained from G. W. Fischer or collected by the authors from cereals and grasses in the Pacific Northwest. The following species were studied: *Tilletia asperifolia*, *T. asperifolioides* G. W. Fisch., *T. bromi-tectorum* J. Urries, *T. caries*, *T. cerebrina*, *T. contraversa*, *T. elymi*, *T. fusca*, *T. guyotiana*, *T. holci*, *T. pallida* G. W. Fisch., and *T. scrobiculata* G. W. Fisch.

All spores used in these studies were removed from the sori, surface-sterilized 1 minute with a 5% solution of Clorox (5.25% sodium hypochlorite), washed in sterile distilled water, and placed on substrata suitable for germination: 2 and 3% water agar, 2% soil-extract agar (8), soil (Palouse silt-loam), and vermiculite. The agar was carried in culture dishes and the soil and vermiculite in preparation dishes. Any deviations will be noted in connection with the results of the experiments involved.

In this paper, optimum temperature for germination is regarded as that temperature at which germination is maximum with minimum incubation.

FACTORS AFFECTING GERMINATION OF SPORES OF TILLETIA CONTRAVERSA.—Attempts to germinate spores of the dwarf bunt fungus (*Tilletia contraversa*) in the laboratory over a three-year period have given inconsistent results. Degree of germination varied not only between accessions but also from year to year with the same accession. For example, the spores of an accession from Wasatch wheat from Kalispell, Montana, failed to germinate, whereas those on Turkey wheat from Nephi, Utah, germinated readily under the same laboratory conditions. Spores of the latter accession germinated well in 1954, but in 1955 failed to germinate under seemingly identical conditions. The technique generally consisted of placing the spores on 2% agar in culture dishes at 5° in an unlighted incubator. Therefore, a study of the influence of certain environmental factors on germination was undertaken to develop a more reliable method of germinating the spores. The factors considered were light and substratum, since these had been shown by other investigators (2, 5, 12) to influence spore germination in European collections of *Tilletia contraversa*.

In the experiments with *Tilletia contraversa*, spores were placed on various substrata and then divided into 2 groups: one was placed in a chamber with a glass lid, so as to admit light (either daylight, artificial light, or both); the other was placed in an identical chamber with light excluded. In both chambers the temperature was generally maintained at 5°, though it occasionally fluctuated between 4° and 9°.

In the first experiment, the spores were placed on 2% agar in culture dishes and on field soil in preparation dishes, and incubated in the chambers for 1 month. Light had little effect on the germination of spores on agar, but increased germination manyfold on soil. Soil apparently stimulated germination in some manner in the presence of light.

In a later experiment, dwarf bunt spores were placed on the following substrata and incubated in the presence and absence of light: 1) 2% water agar in culture dishes; 2) 2% soil-extract agar in culture dishes; 3) field soil in preparation dishes; and 4) vermiculite in preparation dishes. Four containers of each substrate were put in each chamber. Estimates of germination were made after 2 months of incubation. The experiment was run in 1955 and 1956 with current-season spores of the same collection of dwarf bunt (designated No. 32, originally from Nephi, Utah). The results are summarized in Table 1.

It is apparent that maximum germination of spores of *T. contraversa* requires a combination of light and some soil factor or factors. This confirms results obtained in Germany by Gassner-Hoechst (5), Böning, Wagner, and Minckwitz (2), and Baylis (1) in Canada. It is also apparent that spores of the same accession of dwarf bunt may vary in germination in the absence of light and the soil factor if collected in different years.

In these and later experiments, germination was high when the spores of dwarf bunt were placed on soil extract agar in the presence of light. Accessions of spores that germinated only a few per cent on a water agar medium, and then only after prolonged incubation, germinated abundantly in light on soil-extract agar in 1 month to 6 weeks. This greatly facilitated biological studies of dwarf bunt that required large quantities of germinating spores.

The exact nature of the spore-germination stimulant in soil has not been determined. Because it is present in soil-extract agar, which contains the filtrate obtained by pouring boiling water over field soil, the substance is evidently soluble in hot water. Spore germination is more dependable on soil-extract agar than on soil, possibly because of the difficulty of controlling soil moisture. Also, spores on agar can be surface-sterilized and remain free of contaminating organisms during incubation, and are more easily removed for inoculating seeds and seedlings.

TABLE 1.—*Percentage germination of spores of Tilletia contraversa after 2 months' incubation at 5°C on 4 substrata, under conditions of light and darkness.*

Year and substrate	% germination[a]	
	Light	Dark
1955		
Water agar	5	11
Soil-extract agar	69	31
Field soil	1	[b]
Vermiculite	11	[b]
1956		
Water agar	[b]	[b]
Soil-extract agar	75+	[b]
Field soil	75+	[b]
Vermiculite	0	0

[a] Average of 4 replications.
[b] Less than 1%.

TABLE 2.—*Germination of spores of 12 accessions of T. contraversa at 5° in greenhouse or incubator*

Col. no.	Host	% germination[a] at days indicated: Greenhouse 14	20	25	34	44	Incubator 14	20	25	34	44	56
32	Wheat	0	0	[b]	50	75	0	0	[b]	20	25	
29	Wheat	0	0	[b]	50	80	0	0	[b]	30	43	
28	Wheat	0	0	0	10	60	0	0	0	1	1	4
22	Wheat	0	0	1	38	70	0	0	0	2	5	5
196	Wheat	0	0	15	63		0	1	4	8	10	
80	Rye	0	0	[b]	28	63	0	0	[b]	15	35	
180	Rye	0	0	2	58		0	0	[b]	15	20	
181	Rye	0	[b]	15	83		0	0	1	33	40	
190	*F. idahoensis*	0	0	3	88		0	0	[b]	20	25	
193	*A. cristatum*	0	25	73			1	20	28	35		
195	*A. desertorum*	0	8	50	78		1	10	18	65		
189	*A. intermedium*	0	35	48	73		0	1	13	48		

[a] Average of 2 culture dishes.
[b] Less than 1%.

To determine whether the stimulating effect of light and soil-extract agar would extend to other accessions of *Tilletia contraversa*, tests were undertaken with 11 additional collections, some of which had been shown to be capable of only a trace of germination on water agar in the refrigerator. Spores of these accessions, together with the accession used in the experiment just described, were placed on soil-extract agar and divided into 2 groups: one was put in the greenhouse and exposed to diffused daylight; the other was placed in an incubator, where it was exposed to light only occasionally, when the incubator door was opened. The following collections were compared: No. 32, from Nephi, Utah; No. 29, from Pullman, Washington; No. 28, from Kalispell, Montana; No. 22, from Genesee, Idaho; and No. 196, from Worley, Idaho, all from wheat; No. 80, from Flora, Oregon; No. 180, from Bozeman, Montana; and No. 181, from Tetonia, Idaho, all from rye; No. 190, from Elgin, Oregon, from *Festuca idahoensis* Elmer; No. 193, from *Agropyron cristatum* (L.) Gaertn.; No. 195, from *A. desertorum* (Fisch.) Schult. from Worley, Idaho; and No. 189, from *A. intermedium* (Host) Beauv. from Rockford, Washington. The results are shown in Table 2.

Spores of all accessions germinated faster and more completely in the greenhouse than in the comparative darkness of the incubator (Table 2). Those from grasses and rye generally germinated more quickly than those from wheat. This confirms Niemann's (11) observations. Spores of all the accessions from wheat germinated at about the same rate in the presence of light. In the incubator, however, differences were readily apparent in the rate and degree of germination of these accessions from wheat. Of two accessions (22 and 28) that had proved difficult to germinate in earlier studies, only about 5% of the spores germinated in 56 days in the darkened incubator. These two accessions apparently require more light than one such as No. 29, in which 43% of the spores germinated in comparative darkness in the incubator.

It should be pointed out that the accessions of *Tilletia contraversa* from grasses and rye tested were not necessarily pathogenic races distinct from those on wheat. The former were obtained from fields having known histories of heavy dwarf-bunt infestation in wheat, and heavy infection resulted when wheat was inoculated with the spores from grasses and rye. The host species from which the spores were obtained may have influenced the rate of spore germination. However, in the case of wheat and rye, accessions from the same host species also differed somewhat in rate of germination.

FACTORS AFFECTING SPORE GERMINATION OF TILLETIA SPP. OCCURRING ON GRASSES.—1) *Temperature.*—In a preliminary test, spores of 48 accessions of Tilletia representing 12 species were placed on 3% agar in culture dishes and incubated at 5°, 10°, and 15° in

TABLE 3.—*Effect of temperature on the germination of spores of representative accessions of Tilletia bromitectorum from Bromus tectorum*

Acc. no.	Temp. (°C)	% germination at days indicated: 10	14	20	34	44
49	2	0	70	—[a]	—	—
	5	15	85	—	—	—
	10	0	2	2	2	2
50	2	0	0	15	55	—
	5	0	1	20	65	—
	10	0	0	0	0	0
53	2	0	0	0	10	15
	5	0	0	2	25	40
	10	0	0	0	0	0
56	2	0	35	60	—	—
	5	0	65	70	—	—
	10	0	25	30	40	40
62	2	0	0	10	65	—
	5	0	65	75	—	—
	10	0	0	0	0	0
74	2	0	2	75	—	—
	5	5	85	95	—	—
	10	0	12	35	70	—
	15[b]	0	0	1	30	35
93	2	0	0	0	30	55
	5	0	0	0	0	25
	10	0	0	0	0	0

[a] Germination advanced too far for accurate percentage determinations.
[b] All collections were incubated at 15°, but no germination occurred at this temperature except in this accession.

incubators. The spore accessions had been stored in envelopes at room temperature for 4–10 years.

With most of the species, 5° proved to be more favorable for germination than 10° or 15°. Germination at 5° occurred after 2–4 weeks of incubation in *Tilletia caries* from *Agropyron trachycaulum* (Link) Malte, *T. contraversa* from *A. intermedium*, *A. subsecundum* (Link) Hitchc., *Arrhenatherum elatius* (L.) Presl, wheat, and rye; *T. elymi* from *Elymus glaucus* Buckl.; *T. fusca* from *Bromus ciliatus* L., *B. marginatus* Nees, *F. pacifica* Piper, and *F. octoflora* Walt.; *T. asperifolioides* from *Muhlenbergia filiformis* (Thurb.) Rydb.; *T. scrobiculata* from *Poa secunda* Presl; and *T. holci* from *Holcus lanatus* L. Spores of *T. bromi-tectorum* from *Bromus tectorum* L. and *B. rigidus* Roth germinated in less than 2 weeks at 5°.

Spores of *T. guyotiana* from *Bromus japonicus* Thunb. germinated faster at 10°, but had much higher germination percentages at 5°. *T. cerebrina* from *Deschampsia elongata* (Hook.) Munro & Benth. germinated equally well at 5° and 10°, requiring less than 30 days' incubation at either temperature. Optimum temperature for *T. asperifolia* from *Muhlenbergia asperifolia* (Nees & Mey.) Parodi was 10°. *T. pallida* from *Agrostis canina* L. germinated only at 15°.

During the study just described, considerable variation was noted in the time required for germination of a given species at a given temperature. Whether such variation was due to a differential response of species and accessions to temperature could not be determined, because the collections had been stored for varying periods (4–10 years). In addition, some of the species were represented by very few accessions, which may or may not have been typical of the species as a whole.

Therefore, to determine whether the variation encountered was due to temperature alone, 3 species of *Tilletia* for which a number of current-year collections were available were selected for study. Spores of *T. bromi-tectorum* (37 accessions) and *T. guyotiana* (14 accessions) were incubated at 2, 5, 10, and 15°. Those of *T. fusca* (11 accessions) were incubated at 5, 10, and 15° only. The data obtained are too extensive to reproduce in entirety. However, the germination behaviors of representative spore collections of *T. bromi-tectorum* are shown in Table 3.

As exemplified by results obtained with T. bromi-tectorum (Table 3), individual accessions within all three species showed considerable variation in the time required for germination as well as in capacity to germinate at various temperatures. The optimum temperature was 5°, but high germinations were obtained at 2° after longer incubation. With the majority of accessions, no germination occurred at 10° or 15°, and in no case was maximum germination attained at these temperatures. In at least one accession of *T. guyotiana* germination was initiated sooner at 10°, but later reached higher germination percentages at 5° and 2°. Germination of *T. fusca* was lower than that of *T. bromi-tectorum* or *T. guyotiana*, in some cases being only 1% after 57 days of incubation.

In general, spores of *T. bromi-tectorum* germinated

TABLE 4.—*Germination of spores of 9 species of Tilletia at 5° in greenhouse and incubator*

Col. no.	Species	Year col.	Spores % germination at indicated days of incubation[a]											
			Greenhouse						Incubator					
			13	20	25	34	44	56	10	20	25	34	44	56
177	*T. asperifolia*	1956	30	75+					10	75+				
156	*T. bromi-tectorum*	1957	35	70					78	80+				
168	*T. bromi-tectorum*	1956	38	75+					30	75+				
172	*T. bromi-tectorum*	1956	7	95					37	75+				
174	*T. bromi-tectorum*	1956	35	75+					35	75+				
175	*T. bromi-tectorum*	1956	0	20	50	75+			0	[b]	3	75+		
185	*T. bromi-tectorum*	1957	78	80+					75	93				
188	*T. bromi-tectorum*	1957	83	85+					65	93				
T-16	*T. caries*	1957	75+						75+					
79	*T. cerebrina*	1954	0	0	0	0	0	8	0	0	0	5	22	32
86	*T. cerebrina*	1954	0	0	[b]	10	38		0	18	58	80		
176	*T. cerebrina*	1956	12	75					0	1	10	18	20	
28	*T. elymi*	1955	0	0	0	0	0	[b]	0	0	0	0	1	7
73	*T. elymi*	1954	0	0	0	0	[b]	15	0	0	0	0	[b]	1
182	*T. elymi*	1956	0	0	0	0	[b]	[b]	0	0	0	0	[b]	5
150	*T. fusca*	1955	0	22	63	75+			0	5	12	75		
165	*T. fusca*	1956	30	75+					0	8	15	27	50	
166	*T. fusca*	1956	32	75+					0	5	15	70		
183	*T. fusca*	1957	48	75+					0	1	5	35	40	
184	*T. fusca*	1957	[b]	73					0	[b]	1	15	15	
186	*T. fusca* (?)	1957	0	37	58				10	75+				
148	*T. guyotiana*	1957	15	95					2	22	43	75+		
155	*T. guyotiana*	1957	22	95					1	20	33	85		
171	*T. guyotiana*	1956	4	93					0	20	68	75+		
173	*T. guyotiana*	1956	35	95					1	52	65	75+		
109	*T. holci*	1954	0	3	55	75+			0	0	0	5	10	10
179	*T. scrobiculata*	1956	0	0	0	0	0	[b]	0	0	0	[b]	25	40

[a] Each percentage represents the average of 2 plates. No germination at 10 days of incubation except *T. caries*, which showed 60 and 10% in greenhouse and incubator, respectively.
[b] Less than 1%.

faster than those of the other two species. In the majority of accessions of this species, spore germination began within 10 days and reached its maximum in 20 days; the majority of accessions of *T. guyotiana* began germination in 14 days and reached their maximum in 34 days; *T. fusca* was slowest, with germination in the majority of accessions of this species beginning in 20 days and reaching maximum in 34 days.

2) *Light.*—An experiment was performed to determine the effect of light on spore germination in species of *Tilletia* other than *T. contraversa*. Spores of accessions of 9 species of *Tilletia* were placed on soil-extract agar in culture dishes. Two dishes of spores of each species were put in an incubator where they were exposed to light only occasionally when the door of the incubator was opened. A similar number of dishes were placed in the greenhouse and exposed to natural and artificial light, but shielded from direct sunlight. Temperatures in both the greenhouse and incubator were maintained at 5° except for occasional respective fluctuations to 8° and 10°. The spores were examined and percentages of germination estimated at 7 different periods of incubation, ranging from 10 to 56 days. The results are given in Table 4.

The effect of light on spore germination differed with the species tested, and in some cases between accessions of the same species. Light had little apparent effect on *Tilletia asperifolia*, *T. bromi-tectorum*, and *T. elymi*. Light hastened the germination of spores of *T. fusca*, *T. guyotiana*, *T. holci*, *T. caries*, and one accession of *T. cerebrina* (No. 176), but appeared to suppress the germination of *T. scrobiculata* and 2 accessions of *T. cerebrina*. *T. holci* and certain accessions of *T. cerebrina* and *T. fusca* did not reach maximum germination in the incubator during the observation period.

Since spores placed in the incubator were exposed to occasional light, and therefore were not actually incubated in total darkness, an experiment was set up in which half of the spores were incubated in light and half in total darkness in the chambers described previously. Both chambers were placed in the greenhouse at 5° (with occasional fluctuations between 5° and 10°). Duplicate culture dishes of spores on soil-extract agar of each species were placed in each chamber. In addition, one plate remained outside but near the chambers. Temperatures were generally identical in the chambers during the incubation period, but varied occasionally up to 1°. Seven species of *Tilletia* were selected for testing. The results are presented in Table 5.

TABLE 5.—*Effect of light and total darkness on the germination of spores of 7 species of Tilletia*

Species	% germination at days indicated[a]							
	Light				Darkness			
	8	11	16	25	8	11	16	25
T. asperifolia	0	53	75+		0	[b]	3	30
T. bromi-tectorum		0	0	3		0		0
T. bromi-tectorum		[b]	[b]	[b]		**0**		**0**
T. caries	50	75+			18	63		
T. cerebrina		0	0	0		0		0
T. fusca		88	90+			0		5
T. guyotiana		18	43	90		0		0
T. holci		0	1	70		**0**		**0**

[a] Average of 2 culture dishes. Maximum germination (not shown) occurred with all species in the culture dishes that remained exposed to light outside the chambers.

[b] Less than 1%.

These results show that spore germination was almost entirely suppressed when light was excluded entirely during the incubation period, except in *T. asperifolia* and *T. caries*. Even in these, the rate of germination was retarded by the absence of light. The germination of spores of *T. bromi-tectorum* and *T. cerebrina* was also suppressed in the chamber exposed to the light, whereas spores of these species in dishes exposed to light outside the chambers attained maximum germination. The cause of the suppression of germination in the lighted chambers is not known. Spores germinating in total darkness were observed to form elongated promycelia and few primary sporidia.

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TRANSMISSION OF POTATO MOTTLE VIRUS TO, AND FROM, CITRUS PLANTS BY MECHANICAL INOCULATION [1]

Francis O. Holmes

SUMMARY

Potato mottle virus was transmitted with difficulty to healthy seedlings of Mediterranean sweet orange, Orlando tangelo, and Meyer lemon by rubbing inoculum into their leaves in the presence of finely powdered carborundum. It was recovered regularly and with ease by subinoculation to tobacco from chlorotic lesions that developed in the inoculated leaves, but it was not recovered at all from green areas of the same leaves. Earlier failures to transmit mechanically the viruses that occur naturally in citrus plants may be attributable to the use of insusceptible or insufficiently susceptible test plants rather than to any inhibitor in citrus foliage.

Viruses occurring naturally in citrus have proved transmissible in all cases by graft, and occasionally by other biological processes (3), but not by purely mechanical means such as rubbing or puncturing leaves. Thus no adequate method of titrating these viruses is available, but not because of neglect of the problem. Many attempts have been made to transmit by mechanical means the viruses that cause tristeza, psorosis, xyloporosis, and exocortis, both to healthy citrus plants and to a wide variety of test plants that currently serve for the detection and titration of better known viruses. All such attempts seem thus far to have failed, which is surprising in a way, because the virus of tristeza, at least, belongs to the nonpersistent aphid-transmitted group (1), and almost all known aphid-transmitted viruses that are nonpersistent in their insect vectors have proved mechanically transmissible.

The question arises whether attempts at mechanical transfer of citrus viruses have failed because of inhibitors in the infected tissues, a choice of insusceptible or insufficiently susceptible test plants, or inadequate methods of inoculation. The question is difficult to answer. The degree of susceptibility of specific test plants and the effect of possible inhibitors in affected foliage can be appraised only if adequacy of inoculation methods can be established, and vice versa, the adequacy of inoculation methods can be appraised only if sufficiently susceptible test plants are found.

To break this cycle of uncertainty, an attempt was recently made to produce a more easily investigated model system. This was done by introducing experimentally into citrus plants a virus known to be highly infective to conveniently available test plants. It was anticipated that the appropriate local-lesion hosts for such a virus, being already known, would permit appraisal of the adequacy of various methods of inoculation in the presence of extracts of citrus foliage.

Seedlings of 7 citrus species or interspecific hybrid lines were inoculated in approximately equal numbers: Mediterranean sweet orange (*Citrus sinensis* (L.) Osbeck), Orlando tangelo (*Citrus paradisi* Macf. × *C. reticulata* Blanco), Meyer lemon (*Citrus Limon* (L.) Burmann × *C. sinensis* (L.) Osbeck), Key lime (*Citrus aurantifolia* (Christmann) Swingle), sour orange (*Citrus Aurantium* L.), trifoliate orange (*Poncirus trifoliata* (L.) Raf.), and Mauritius papeda (*Citrus hystrix* DC.).[2]

Juices expressed from stock plants of Turkish tobacco (*Nicotiana tabacum* L.), each carrying one of three viruses of wide host range, were used as inocula. Each inoculum was placed on a cheesecloth pad, which was then rubbed over foliage previously dusted lightly with powdered carborundum of 320- or 600-mesh grade. The viruses were tobacco mosaic virus (*Marmor tabaci* H.), tobacco ringspot virus (*Annulus tabaci* H.), and the ringspot strain of potato mottle virus (*Annulus dubius* H.). Only the last gave evidence of infecting some of the plants. Throughout the experiment, citrus plants of the kinds inoculated, in equal or larger numbers, were held as controls without inoculation on the same greenhouse bench. None of these showed evidence of any viral disease.

Three single lesions, and only three such lesions, were produced as a result of inoculations with potato mottle virus. They were first noticed 6, 6, and 7 months after the dates of inoculation. It is still uncertain how soon the lesions would have been visible enough to have been found if leaf-by-leaf examinations had been made regularly after the first few weeks following inoculations. When found, the lesions were conspicuous (Fig. 1) and alike in being large (½ in. in diameter or more), nearly circular, chlorotic spots displaying concentric bands of yellowish green color. These lesions crossed side veins without difficulty, but one appeared to be limited in lateral spread by a midvein. They did not extend themselves conspicuously along veins or show any evidence of extension to other leaves or to distant parts of the infected plants. Not only did the tops of the infected plants remain symptomless but 4 leaves selected from each for testing by subinoculation failed to disclose the presence of any virus.

The first plant found infected was a seedling of Mediterranean sweet orange; the second, of Meyer lemon; the third, of Orlando tangelo. This scattering of infection in 3 of the 7 inoculated kinds of citrus suggested that no kind was especially susceptible.

[1] Accepted for publication May 5, 1959.

[2] Seeds and seedlings of these species and varieties were supplied by the Citrus Experiment Station of the University of Florida at Lake Alfred, Florida, through the kindness of Dr. L. C. Knorr.

Possibly, some later modification of the method of inoculation may improve results, but no better procedure has yet been found. Rubbing the upper leaf surface in the presence of carborundum powder and of undiluted inoculum was the only procedure that seemed effective enough to produce any lesions at all. Buffers in the inoculum did not improve results. Variations of wounding procedure, such as pin-pricking, leaf-edge rubbing (5), and preinoculation darkening of plants proved ineffective. Heat treatment of citrus foliage before inoculation, as used by Yarwood (6), did not appear helpful. The first lesion produced by rubbing followed the inoculation of all available leaves of 18 plants, including representatives of all the seven types of citrus previously mentioned; the second, the inoculation of 17 plants; the third, the inoculation of 43 plants.

Virus was recovered with ease from the lesion in the Mediterranean sweet orange by excising part of the affected tissue, crushing it, and inoculating 2 plants of Turkish tobacco and 1 of *Nicotiana glutinosa* L. All 3 of these plants became systemically infected. Four additional small parts of the same chlorotic lesion were later tested by subinoculation to 4 young plants of Turkish tobacco. Controls consisted of subinoculations from small leaf areas originally located in the same inoculated Mediterranean sweet orange leaf about $\frac{3}{8}$–$\frac{5}{8}$ in. from the chlorotic lesion. The subinoculations from the lesion produced 42, 22, 19, and 8 primary lesions, respectively, in the Turkish tobacco plants, all of which showed systemic disease by the 10th day after inoculation. The 4 control inoculations produced 0, 0, 0, and 0 primary lesions, and no systemic disease. This indicated that the virus recovered on subinoculation was not a residue from the inoculation process but was virus proper to the infected area of the leaf.

Virus was also recovered from the lesion found in a seedling of Meyer lemon. Four Turkish tobacco plants were inoculated from affected tissue, and 4 other Turkish tobacco plants were inoculated from unaffected tissues nearby in the same lemon leaf (about $\frac{1}{4}$–$\frac{1}{2}$ in. away from the chlorotic area). Lesion counts were 15, 27, 30, and 28 for subinoculations from the lesion area; they were 0, 0, 0, and 0 for control subinoculations from nearby green areas in the inoculated leaf; and 124 for subinoculation from a Turkish tobacco stock plant carrying the same virus.

Virus was recovered from the lesion in a plant of Orlando tangelo by similar subinoculation. All of 4 young Turkish tobacco plants thus inoculated became infected, showing 75, 105, 111, and 114 primary lesions, respectively. Control plants receiving the same kind of inoculation, but with inoculum from nonlesion areas about $\frac{1}{2}$ in. from the observed lesion in the inoculated tangelo leaf, all remained uninfected.

Success in recovering the introduced virus upon each attempt at mechanical transmission from the affected areas is instructive. It suggests that the difficulty consistently experienced in attempts to transfer naturally occurring viruses, including those of tristeza and psorosis diseases, has not been caused by inhibitory substances in the affected foliage. Neither is there any indication in the present experiments that transfer of viruses from citrus tissues would require special techniques of subinoculation. On the other hand, the difficulty of establishing potato mottle virus in inoculated citrus leaves, even when the inoculum contains a high titer of the virus and the virus has been proved capable of multiplication and lesion formation after it is once established, suggests that the first objective in future work must be the discovery of more receptive test plants or still further increases in the titers of inocula.

The present finding that potato mottle virus can be established experimentally in citrus tissues raises the question whether it may also become established in such tissues from time to time under natural condi-

Fig. 1. Unsuccessfully and successfully inoculated leaves of Meyer lemon seedling. Lesion produced by potato mottle virus on leaf at right, 180 days after inoculation. Natural size. Photograph by J. A. Carlile.

tions in the field, and whether mutants of this virus capable of systemic movement in citrus plants may have arisen and become widespread somewhere in nature. It seems desirable to search for mutant strains of potato mottle virus in long-established plantings of citrus trees.

Citrus plants experimentally infected by potato mottle virus may provide a model system for the study of purification methods for naturally occurring citrus viruses. Moreover, potato mottle virus is such a good antigen (2) when introduced into experimental animals, and gives such good protection in plant hosts against reinfection by other strains of the same virus (4), that a variety of serological and immunological tests would be feasible with such a model system to determine whether infected citrus tissues present unusual problems in these areas of investigation.

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SYMPTOMS AND HOST-PARASITE RELATIONS OF THE ALTERNARIA LEAFSPOT DISEASE OF CUCURBITS [1]

Curtis R. Jackson [2]

SUMMARY

Symptoms of Alternaria leafspot on muskmelon, watermelon, cucumber, and squash differ in size, shape, and color of lesions and in rate of disease development. Sun-scalded muskmelon and cucumber fruit are invaded by the pathogen. The viability of conidia was unimpaired in 8 months of storage *in vitro* at constant temperatures of 8, 28, and 36° C. Conidia were not recovered from leaf debris that had been buried in the field, although mycelium of the pathogen was present and conidia developed when unearthed leaf fragments were placed in a moisture chamber. Conidia produced by mycelium in dead host material are believed to be the primary inoculum for spring infections. Seedborne conidia may be important in introducing the pathogen into new areas. Serious outbreaks in Florida occur when daily temperatures are in the range of 68–90°F. Germination hyphae of the pathogen penetrate the host leaf directly, and lesion enlargement results from intercellular growth of hyphae and subsequent destruction of all leaf tissues.

The Alternaria leafspot disease, incited by *Alternaria cucumerina* (E. & E.) J. A. Elliot, is found throughout the world on various cucurbits. The most frequently reported hosts are muskmelon and watermelon, but other cucurbits are also attacked (5). Information is presented herein on host-parasite relations derived from experiments with muskmelons.

SYMPTOMS.—*Muskmelon.*—The earliest symptoms appear on the upper leaf surface, as yellow or brown flecks, 0.5 mm in diameter, surrounded by light green halos. Lesions develop distinct concentric zones, giving a target-board appearance, but under conditions highly favorable for development of the pathogen this zonation is often lacking. Individual lesions enlarge to 5–20 mm and become light to dark brown. Marginal necrosis and coalescence of lesions are common, resulting in large, irregular necrotic patches. Lesions become torn and ragged with age but seldom approach a shot-hole condition. Sun-scalded or overripe fruits are occasionally invaded by the pathogen.

Watermelon.—Symptoms begin with the appearance on the upper leaf surface of tan flecks, 0.2–0.5 mm in diameter, surrounded by pale green to bright yellow halos. Disease development is usually slow, and lesions become dark brown to black while less than 2 mm in diameter. The halos later become indistinct, and zonation is frequent. Lesions become 1–10 mm in diameter, and confluent marginal necrosis is common.

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Fruit infection has not been noted by the writer, but has been reported by Nattrass (8).

Cucumber.—Initial leaf symptoms are bright to pale yellow spots 1.0–1.5 mm in diameter. Disease development is slower than on muskmelon, and, as lesions expand, the necrotic tissue becomes light brown without evident zonation. Older necrotic tissue may become slightly raised and shield-like, with a distinct darkening of the veinlets, giving the lesions a netted appearance. Lesions become 1–12 mm in diameter and are circular or irregularly lobed. Infection of sunscalded fruit is essentially similar to that of muskmelon.

Squash.—The 3 species and various varieties of *Cucurbita* into which common squashes are grouped exhibit similar symptoms. Leaf lesions are initially pale yellow and 0.5–1.0 mm in diameter. Disease development is rapid and chlorotic areas enlarge and frequently become blister-like. Lesions become tan to brown, 1–12 mm in diameter, and often with dark brown borders. Marginal necrosis and confluence of lesions are common.

HOST-PARASITE RELATIONS.—*Longevity of the fungus.*—The longevity of conidia *in vitro* was investigated by storing Petri-dish cultures of the fungus at different constant temperatures and assaying the viability of conidia periodically. Dry cultures of each of 2 isolates were prepared by culturing the fungus on V-8 juice agar medium for 20 days at 28°C and then drying the cultures at 32°C. Dry cultures were stored at −8, 8, 24, 28, 36, and 44°C. The viability of conidia of each isolate was determined before and after storage by removing a sample of conidia with a small, dry brush, placing them on water agar for 10 hours at 28°C, and counting the number germinated in a random inspection of 200 conidia.

The viability of both isolates was above 98% before storage. The results indicate that the viability of conidia was unaffected by 8 months' storage at 8, 28, and 36°C. Fewer than 5% of the conidia stored at −8°C were viable after 8 months and viability in the 24° and 44°C groups was reduced below 45% in 8 months.

Sources of inoculum.—Brisley (1) believed that mycelium living in dead vines supplied spring inoculum, and Martin (6) suggested that the pathogen persisted in dead vines since the disease was most severe in fields repeatedly planted with melons. Van Haltern (9) presented experimental evidence that the disease developed rapidly when muskmelons were replanted following a diseased crop. Using untreated seed artificially infested with conidia, he also found that an increase in disease incidence was related to seed-borne conidia. Middleton and Whitaker (7) suggested that seed-borne conidia are responsible for early infection of crown leaves.

To investigate the ways in which the fungus survives or is introduced, groups of 8–10 severely infected muskmelon leaves were secured together in ¼-in. hardware-cloth packets. Several packets were placed on the soil surface in a small field plot; others were buried at depths of 6 and 9 in. The packets were set out in June and unearthed 8½ months later. Small compacted masses of leaf fragments were recovered from the buried packets, whereas only fragments of petioles remained in the surface packets. Soil and leaf fragments of four packets from each depth were blended with 500 ml water. The resulting suspension was examined microscopically for conidia, and sprayed on mature cantaloupe plants and watermelon seedlings. In addition, some compact masses of leaf fragments were carefully examined and then placed in small moisture chambers at 28°C.

Although conditions were favorable for disease development and plants were repeatedly inoculated with leaf debris suspensions, no infection resulted. Newly formed conidia of the pathogen were observed along the edges of the leaf fragments after 60 hours in moisture chambers. The identity of the conidia was verified by inoculation tests.

In a second experiment, cantaloupe seeds were disinfested with mercuric chloride, rinsed and dried, and divided into 2 equal lots. One lot was infested by dusting with conidia of the pathogen. Equal numbers of seed of each lot were planted in autoclaved sand, after 4 months of storage at room temperature. There was no evidence of root or hypocotyl infection in plants of either lot. Four weeks after planting, small brown lesions were observed on the cotyledons of 4% of the 115 plants from infested seed, and the pathogen was isolated from these lesions. Seedlings from uninfested seed did not become infected.

In the greenhouse, conidial inoculum was consistently effective in inciting the disease, but the use of suspensions of mycelial fragments and carefully controlled temperature and relative humidity never resulted in infection. Conidia are believed to be necessary for primary spring infections. The almost complete absence of conidia on decayed leaf tissue and in surrounding soil strongly suggests that conidia of this fungus are unable to survive in a soil environment and probably have no important function in overwintering of the fungus in the field. Since the fungus has been shown to persist in decaying leaf material, the primary conidial inoculum results from renewed growth of the mycelium in host debris, when field conditions become favorable, and subsequent production of conidia. This conclusion was partially substantiated by observations during the early spring in fields previously planted with muskmelons. Although the fields had been cleared and planted with oats several months before, fragments of muskmelon vines and leaves were found that harbored mycelia of the pathogen and other fungi. Conidia of the pathogen developed readily when these vine and leaf fragments were placed in a moisture chamber.

The pathogen can be introduced into new areas through seed infested with conidia since these conidia can endure seed storage periods and later infect cotyledons of the host, leading to limited production of new conidia.

Climatic conditions favoring natural infection.—Chupp (2) noted that unusually abundant rainfall and warm weather preceded a severe outbreak of the dis-

ease in New York, and Godfrey (4) reported an epiphytotic during the dry season in Texas. Weber and Owen (10) observed heavy infection of watermelon in central Florida during an unusually dry spring and summer.

From a recording station within 200 yards of muskmelon variety plots, climatological data were obtained for 2 seasons during which infection and disease development were rapid. Initial infection of muskmelon occurred during September, 1956. Mean minimum and maximum temperatures for the month were 67 and 88°F and total rainfall, distributed among 12 days, was 3.9 inches. In May, 1957, when the disease was again noted in its initial stage on a second crop, mean minimum and maximum temperatures were 63 and 85°F, with 8.6 inches of rainfall distributed among 17 days. Mean minimum and maximum relative humidities for May were 43 and 99% with a mean daily duration of maximum relative humidity of 9.2 hours. The total amount of rainfall is apparently less important than the daily distribution of rainfall and dew. Field observations indicate that the disease occurs in severe proportions only when daily temperatures are 68–90°F.

Host penetration and pathological anatomy.—Host penetration was studied in a manner detailed by Diener (3). For a study of the effects of the pathogen on the host leaf anatomy, paraffin sections of lesions in various stages of development were prepared and stained with safranin and fast green.

The host leaf epidermis was penetrated directly by the germination hyphae of the fungus. The terminal portions of the hyphae always enlarged prior to penetration. In some cases the hyphal tips continued to elongate after the formation of swollen segments, giving germination hyphae a knotted appearance.

Examination of serial sections of young lesions revealed that, after penetration, hyphae branched and elongated in a radial and downward manner, proceeding intercellularly and intracellularly. The advance into adjacent uninfected tissue occurred by intercellular growth between the upper epidermis and the palisade parenchyma, and concomitantly, between the lower epidermis and the spongy parenchyma. Hyphae penetrated palisade parenchyma cells at their upper ends and eventually destroyed cell contents and many cell walls, leaving small cavities and an irregular network of cell wall fragments. Invasion of spongy parenchyma resulted in destruction and collapse of this tissue. In each instance, epidermal cells were also invaded. Necrotic tissue became compacted with age so that lesions were depressed.

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FREE AMINO ACIDS IN THE CULTURAL MEDIA AND CELLS OF FUSARIUM SOLANI F. PHASEOLI [1]

Charles W. McAnelly [2]

SUMMARY

The free amino acid content of 20 isolates of *Fusarium solani* f. *phaseoli* was examined by paper chromatography, and 11 free amino acids were identified: aspartic acid, glutamic acid, glycine, alanine, lycine, valine, leucine and/or isoleucine, phenylalanine, threonine, gamma-amino butyric acid, and glutamine. Relative quantitative estimations of the amino acid contents of the exudates and cells of 10 of the isolates were obtained by area determinations under individual amino acid peaks of optical density curves. The curves were plotted from optical density determinations made at regular intervals along one-dimensional chromatograms of cultural filtrates and of cellular extracts of the isolates. The aspartic acid, glutamic acid, glycine, and alanine contents of the cells of 4 weak pathogens were compared to those of 4 strong pathogens. The glutamic acid, glycine, and alanine contents of the weak ones were significantly greater at the 1% level. There was no significant difference between strong and weak pathogens in amounts of aspartic acid. The free amino acid contents of the cultural media of weak and strong pathogens were variable. No particular trends were noted.

Little is known of the biochemistry of fungi pathogenic to plants. A greater knowledge of the processes and products of synthesis of these organisms would materially aid in understanding the physiological relations between host and parasite.

Paper chromatography has proved an effective research tool in separating and identifying many chemical compounds. Only a few workers have used this tool as an aid to understanding the basic differences between the genera, species, races, or strains of pathogenic fungi. Broyles (2) studied the sugar and amino acid composition of uredospores of races of cereal rusts with this technique and found qualitative and quantitative differences among the races. Studies of the amino acids of strains and species of pathogenic fungi have been made by other workers (4, 7, 8).

The present investigation was undertaken to determine if free amino acids synthesized by strains of *Fusarium solani* (Mart.) Appel. & Wr. f. *phaseoli* (Burk.) Snyd. & Hans. differ qualitatively and quantitatively.

METHODS AND MATERIAL.—Twenty Fusarium isolates that had shown various abilities to produce root rot in beans under greenhouse conditions were selected for this study. For several months the cultures were maintained on nutrient agar slants composed of distilled water (1000 ml), sodium nitrate (2.0 g), dibasic potassium phosphate (1.0 g), potassium chloride (0.5 g), magnesium sulphate (0.5 g), ferrous sulphate (0.01 g), sucrose (30.0 g), and Difco Bacto Agar (20.0 g). Each was transferred under aseptic conditions to Petri dishes containing the same medium. After a few days' growth, hyphal tips were removed from each culture and transferred to separate 250-ml Erlenmeyer flasks containing 100 ml of sterile nutrient solution. For qualitative determination of the free amino acids, these were allowed to grow 2 weeks under agitation at room temperature. The mycelia and spores were then removed from the liquid by filtration and rinsed with sterile distilled water.

For relative quantitative estimations, 10 of the isolates were grown in the same manner. Those selected were the 5 most virulent and the 5 least virulent pathogens. However, at the end of the first week, 10 ml of each culture was removed and filtered. The filtrate was frozen immediately. At the end of the second week, 20 ml of each culture was removed and used to again test each organism's ability to infect bean plants. After the remainder of the culture was filtered, 10 ml of each filtrate was frozen. All filtrates were later evaporated in a water bath to 1/10 volume before being examined for amino acids.

For the determination of the free amino acids contained within the cells of the organisms, 500 mg of the wet mycelia and spores were ground in 80% ethanol. Each culture was ground for 10 minutes in a small plastic piston-type grinder. The ground material was placed in a small aluminum tray and dried overnight. Twelve hours later 50 mg of the dried material was ground for 10 minutes in 1 ml of 80% ethanol. All of the cells were found to be broken by this treatment. Solids were removed by centrifugation. The liquid was decanted into a vial, evaporated in a water bath to near dryness, and brought to 1/10 of the original volume with 95% ethanol.

For the qualitative determinations and the relative quantitative estimations of the free amino acid content of the exudates and cells of the isolates, the technique of paper-partition chromatography was followed. This procedure was devised by Consden et al (3). Ascending paper chromatography was used as described by Williams and Kirby (10).

Two-dimensional ascending chromatography was used for qualitative determinations of the free amino acids. The solvent for the first dimension was Merck

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reagent-grade phenol and water (80:20). Two beakers, one containing, 0.3V ammonium hydroxide and the other sodium cyanide crystals, were placed in each jar. The solvent used for the second dimension was water-saturated 2,4-lutidine.

Sheets of Whatman Number One filter paper, 20×20 cm, were used. For the two-dimensional chromatograms, two 3-microliter drops of the material to be tested were deposited at one place 2 cm from the bottom of the paper and 2 cm from one edge. The first drop was allowed to dry before the second was applied. The papers were rolled into cylinders and held together with small pieces of Scotch tape. The chromatograms were developed in each solvent at controlled room temperatures to within 2 cm of the top of the papers. The nin-hydrin (1,2,3-triketohydrindene) sensitive compounds were revealed by spraying the sheets with 0.1% of this material in 95% ethanol (9). Using essentially the methods of Dent (5, 6), the spots were identified by known amino acids run at the same time, by their R_f values, their patterns on the chromatograms, and their color.

For relative quantitative determinations, one-dimensional chromatograms were developed in the same manner in phenol and water. Five samples were placed on a paper. Two papers completed a set of samples. Eight replications were made of each set. The spots were placed 2 cm from the bottom of the paper and 3 cm apart. Three microliters were placed per spot with the use of a 1-microliter pipette. Each aliquot was allowed to dry before the next was placed. In a few instances the quantity applied was insufficient for the development of satisfactory chromatograms. For these, the quantity was doubled (6 microliters for each spot). When development was completed, the papers were removed from the jars, dried overnight, and sprayed with ninhydrin.

Relative densities of the ninhydrin-sensitive material were determined 18 hours after spraying by the use of a Welch Densichron. Optical density readings were made every one-half cm from the placement of the material through the 16 cm of travel of the solvent. Average readings of 8 such strips were used to plot optical-density curves. The densities were plotted on the vertical axis against the distance traveled on the horizontal axis. Areas under each peak were determined by the use of a planimeter. When double amounts of the material had been used in order to obtain adequate patterns, one-half the area determination was used as the value. Block (1) determined that areas under density curves are proportional to the concentration of the amino acids.

By comparing the one-dimensional chromatograms with the two-dimensional chromatograms it was apparent that certain of the one-dimensional spots contained 2 ninhydrin-sensitive compounds—alanine and glutamine in one spot, and gamma-amino butyric acid and valine in others. In one instance lysine was present in such amounts that it appeared with valine and gamma-amino butyric acid as one spot.

Relative proportions of the compounds occurring together as one spot were determined by two-dimensional chromatograms of the concentrates in which this occurred. However, the second dimensions were run in 77% ethanol in distilled water. In this solvent the rate of migration of the solvent was much faster than in lutadine, although the patterns developed were comparable.

A determination was made of the maximum optical density of each spot, as well as the area. These two values were multiplied together to give a factor. From the factors, the proportionate amount of each amino acid was determined. The area times the maximum color density of a spot is a function of the amino acid concentration (1). An average of the three such determinations was used to calculate area amounts or percentage of each material under a compound peak.

RESULTS.—Examination of beans grown in soil inoculated with suspensions of mycelia and spores of each isolate revealed that isolate 9, originally a strong pathogen, had lost a considerable amount of its ability to infect beans. The isolates were rated as 4 strong pathogens and 6 weak pathogens.

A total of 11 amino acids were identified in the ethanol extracts of cells of the 20 *Fusarium* isolates. One spot occurring on the chromatograms of one isolate was not identified. The amino acids were: aspartic acid, glutamic acid, glycine, alanine, lysine, valine, leucine, phenylalanine, threonine, glutamine, and gamma-amino butyric acid. Since leucine and isoleucine are not separated by the solvents used, the designation leucine may be leucine and/or isoleucine.

Five amino acids were identified in the ethanol extracts of all 20 isolates. These were aspartic acid, glutamic acid, glycine, alanine, and valine. Leucine was present in all but two of the extracts. Lysine was found in four. Threonine was evidenced in two of the extracts, as was phenylalanine. Gamma-amino butyric acid was present in 15 extracts, and glutamine in nine.

At the end of one week, the cultural media in which the 10 isolates were grown yielded a small amount of a few amino acids. No attempt was made to estimate quantities. Glutamic acid was detected in all of these. Alanine was identified in the media of four weak pathogens and in those of two of the strong ones. Aspartic acid was found in only one, that of a weak pathogen. Gamma-amino butyric acid occurred in the media of two weak and two strong pathogens. Lysine was found in the media of one strong pathogen.

At the end of the second week the cultural media of the 10 isolates yielded a number of amino acids in considerable amounts. Aspartic acid was found in 7 of these samples. Two were from strong pathogens and five were from weak ones. Two samples from strong pathogens, and one from a weak pathogen, contained no detectable amounts of this acid.

The media of all isolates contained various amounts of glutamic acid, alanine, valine, and leucine. Glycine was present in the media of seven. Lysine was found in eight of these, but was no longer found in that of isolate 7. Gamma-amino butyric acid was detected in three samples, and glutamine in one of them. Gamma-amino butyric was no longer found in the cultural

TABLE 1.—*Relative quantitative estimates*[a] *of the free amino acids in the cultural media of 10 isolates of Fusarium solani f. phaseoli at the end of 2 weeks*

Amino acid	1	2	3	4	5	6[b]	7[b]	8[b]	9	10[b]
Aspartic acid	8.9	3.0	5.0	2.3	3.0	1.5		1.0		
Glutamic acid	9.5	4.4	7.2	6.1	3.4	3.7	10.0	3.4	19.0	6.4
Glycine	10.0	1.0		3.7	2.5			2.5	2.2	2.2
a-alanine	16.7	5.7	5.6	2.7	12.0	2.6	19.9	14.0	12.5	23.5
Lysine	29.0	3.2	20.2	26.3	7.6	7.8		8.9		5.0
Valine	2.3	1.8	10.0	8.3	2.5	5.0	2.1	9.5	13.0	5.5
Leucine	6.0	1.3	3.8	2.4	.8	3.3	1.6	1.0	1.1	.5
α-amino butyric acid	18.2						17.5	10.1		
Total	100.6	20.4	55.7	45.8	31.8	23.9	51.1	50.4	47.8	43.1

[a] Values are derived from area determinations under each peak of the optical-density curves of the amino acid patterns.
[b] Strong pathogens.

media of isolate 4. The relative quantitative estimations of the amino acid content of the cultural media are shown in Table 1.

Table 2 shows the relative quantities of the amino acids found in the ethanol extracts of the cells of the 10 isolates. The extracts of the four strong pathogens yielded smaller total amounts than did those of the weak pathogens. Aspartic acid, glutamic acid, glycine, alanine, valine, and leucine were found in all of the extracts. The greatest amount of aspartic acid contained in any extract of a strong pathogen was equal to the least amount found in a weak pathogen. The alanine content of all of the cell extracts of the strong pathogens was less than that of all but one of the weak ones. The least amount of glycine found in any of the weak pathogens was slightly less than the greatest amount found in the strong ones.

Glutamic acid was present in the greatest quantities in five of the weak pathogens. The other, isolate 1, contained a smaller amount than two of the strong pathogens, and the same amount as one other. The amount of valine and leucine was quite variable. Some of the weak pathogens contained more, and some less, than the strong ones. Different amounts of gamma-amino butyric acid were found in 7 of the extracts. Glutamine was detected in 5 of the samples.

The aspartic acid, glutamic acid, glycine, and alanine contents of 4 strong pathogens were compared with 4 weak pathogens. Isolates 1 and 9 were omitted. These two were considered to be atypical, because of known change in pathogenicity. The glutamic acid, glycine, and alanine content of the weak pathogens was significantly greater at the 1% level than were those of the strong pathogens. There was no significant difference in amounts of aspartic acid between classes of pathogens. These data are shown in Table 3.

DISCUSSION.—The cells of the strong pathogens were found to contain smaller amounts of detectable free amino acids. This may be correlated with the tendency of their aspartic acid, glutamic acid, glycine, and alanine content to be less than those of the weak pathogens. The relative amounts of valine and leucine were variable. The greatest and least amounts of these were found in extracts of weak pathogens. The separation of leucine and isoleucine might have indicated some trend.

Less error was introduced in the determination of the relative quantities of aspartic acid, glutamic acid, and glycine. These spots were distinct and well separated. Some of the others required a second determination, which introduced additional factors of error.

The amounts and kinds of the amino acids found in the cultural media at the end of the two-week period were variable. The media of only two of the strong pathogens contained aspartic acid, whereas the other two contained the greatest amounts of alanine found in any of the isolates. The filtrates of weak pathogens, except for number 9, all contained aspartic acid.

TABLE 2.—*Relative quantitative estimates*[a] *of the free amino acid content of ethanol extracts of ground cells of 10 isolates of Fusarium solani f. phaseoli*

Amino acid	1	2	3	4	5	6[b]	7[b]	8[b]	9	10[b]
Aspartic acid	.8	1.0	2.3	2.0	1.5	.6	.6	.5	3.4	.8
Glutamic acid	1.0	3.7	5.2	4.3	3.3	.8	1.4	1.6	11.5	1.0
Glycine	1.4	4.2	5.0	7.0	3.3	.7	1.5	1.0	3.8	1.3
a-alanine	3.1	8.9	8.8	6.5	11.8	2.2	3.2	2.0	13.5	2.5
Lysine				5.5	1.8					
Valine	.2	.1	10.2	14.7	1.4	.9	.4	.5	7.0	2.8
Leucine	.6	1.5	2.0	2.0	.2	1.4	.8	.4	5.4	1.0
α-amino butyric acid	5.1	5.9			2.1	2.1	2.5	2.5	3.7	
Glutamine		3.8	.2		2.2			1.4	7.3	
Unknown						1.5				
Glutamine			3.9							
Total	12.2	29.1	33.7	42.0	27.6	10.2	10.4	9.9	55.6	9.4

[a] Values are derived from area determinations under each peak of the optical-density curves of the amino acid patterns.
[b] Strong pathogens.

TABLE 3.—*Relative total free amino acid content of 4 weak and 4 strong pathogens*

Amino acid	Area units (cm^2)		
	Weak pathogens	Strong pathogens	Difference
Aspartic acid	1.70	.62	1.08
Glutamic acid	4.12	1.20	2.92**
Glycine	4.88	1.12	3.76**
a-alanine	9.00	2.48	6.52**
Average total	4.92	1.35	−3.57
Weak versus strong pathogens			
LSD at 5% = 1.11			
LSD at 1% = 1.50			

The filtrates and extracts were examined as near to the original state as was possible. The concentration of the solutions was necessary to obtain adequate chromatographic patterns. Other amino acids were probably present in quantities too small to detect by the methods used in this study.

It was not surprising to find that one of the strong pathogens had lost its ability to cause severe root rot of beans. Isolate 9 had been tested in the greenhouse, for the first time, 5½ months earlier. It had been rated a strong pathogen. In later tests this isolate demonstrated little ability to infect beans. The loss of such ability has been demonstrated on a number of occasions. This may be explained by the fact that some isolates mutate and the mutations tend to be less pathogenic.

The fact that cells of weak and strong pathogens of *F. solani* f. *phaseoli* were found to differ significantly in their glutamic acid, glycine, and alanine content suggests that there may be a relation between these acids and the organism's ability to behave as a pathogen. Studies of the amino acid content of a large number of isolates are now being conducted. The acid hydrolysates of the cultural media and the cellular structures are also being examined for these acids. The amino acid content of healthy and infected bean plants will be determined and compared. From these studies a better understanding of the host-pathogen relationship may be obtained.

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DEVELOPMENT OF THE GRAPE DEAD-ARM DISEASE [1]

T. S. Pine [2]

SUMMARY

On *Vitis vinifera* varieties of grapevines in California the dead-arm disease, caused by *Phomopsis viticola*, is first noted as a leaf spotting, followed by a leaf chlorosis and deformity, and then the appearance of both pycnidia and sterile lesions on canes, petioles, tendrils, and clusters. Infected canes first darken, but eventually bleach, leaving black pycnidia prominently displayed along the canes. The primary source of inoculum is from pycnidia on spurs and clusters remaining on dormant vines. Spring rains spread pycnidiospores to unfolded buds. Infection is accomplished as long as water is present on the leaf surfaces or trapped in the bud folds. A secondary source of infection is slow mycelial growth from diseased to healthy portions of vines. The pathogen penetrates through wounds of any sort, including punctures commonly caused by insects and epidermal openings left when leaf hairs are broken off. Evidence strongly suggests that the pathogen can also penetrate directly through leaf and cane stomata. Corky abrasions on canes, petioles, tendrils, and cluster stems are derived from the coalescing of stomatal-area infections. Tissues beneath points of infection become progressively disorganized until the cells collapse. The characteristic dead-arm condition is obtained when fungal growth girdles the arm. None of the European grape varieties examined in California have shown resistance to attack by *P. viticola*.

INTRODUCTION.—Several workers have studied the dead-arm disease of grapevines as it occurs on American varieties grown in the eastern section of North America (1, 2, 3, 4, 5, 9, 10, 12). Until recently, no such investigation had been conducted in the grape-growing areas of California, where European varieties (*Vitis vinifera* L.) are cultured extensively. The dead-arm disease has increased in importance in California during the past decade. It now causes severe losses in the table-grape region of the northern San Joaquin Valley and is spreading into wine-grape vineyards elsewhere in the State. *Phomopsis viticola* Sacc., the causal organism, and revisions in the control program have been described (6, 7). This paper deals with observations and research on the development of the disease on *V. vinifera* in California.

SYMPTOMATOLOGY.—The first symptom on *V. vinifera* in California appears on new leaf growth as a spotting that often coalesces and necroses. Leaves become progressively yellowed, crinkled, rolled, and tattered, depending on the degree of infection (Fig. 1-A). Necrotic areas along veins are common. The distorted leaves generally remain attached to the shoots. These leaves are usually only at the basal 6–8 nodes of new shoots. This phase of the disease soon becomes hidden, passing unnoticed since, in average seasons, growth beyond the eighth node seldom shows symptoms. Suckering is common at the base of spurs killed by the fungus, giving the vine a bushy appearance.

About 30 days after spring growth starts, the lower internodes of infected shoots begin to develop reddish-brown bodies that appear similar to erumpent fungus fruiting bodies. When present in large numbers, they merge to produce a distinctive corky abrasion (Fig. 1-B). At the same time, dark epidermal spots and small black pycnidia begin to appear on portions of shoots, petioles, tendrils, and cluster stems, generally at random, but sometimes arranged longitudinally along the shoot axis. The cortex typically splits as the shoots mature, with the splits containing and being surrounded by numerous pycnidia (Fig. 2-C). The host epidermis in the pycnidial area darkens, becomes bluish, and eventually bleaches so that the fruiting bodies stand in bold contrast to the epidermis (Fig. 2-B).

When rain or heavy dews are present, some of the berries become infected before harvest. The surfaces of such berries are covered with pycnidia, and the fruit rapidly shrivels and rots (Fig. 2-D).

The fungus spreads through the canes during the dormant season. When a cane or arm is girdled by fungus growth, the characteristic "dead-arm" condition is reached (Fig. 2-A).

INOCULUM FOR INFECTION.—The pycnidia on canes, spurs, petioles, and clusters remaining on the dormant vine provide the primary source of inoculum. When wet, the pycnidia of *P. viticola* exude their spores in long tendrils, and the spores can be washed or splattered over nearby plant parts. In April, 1956, 4-in. pieces of dormant Tokay canes covered with pycnidia were suspended above healthy Tokay cuttings growing in a lathhouse at Davis, California. This arrangement was left undisturbed until May, 1957. At that time all the plants showed both leaf and cane symptoms of dead-arm. Pieces of diseased canes were also attached

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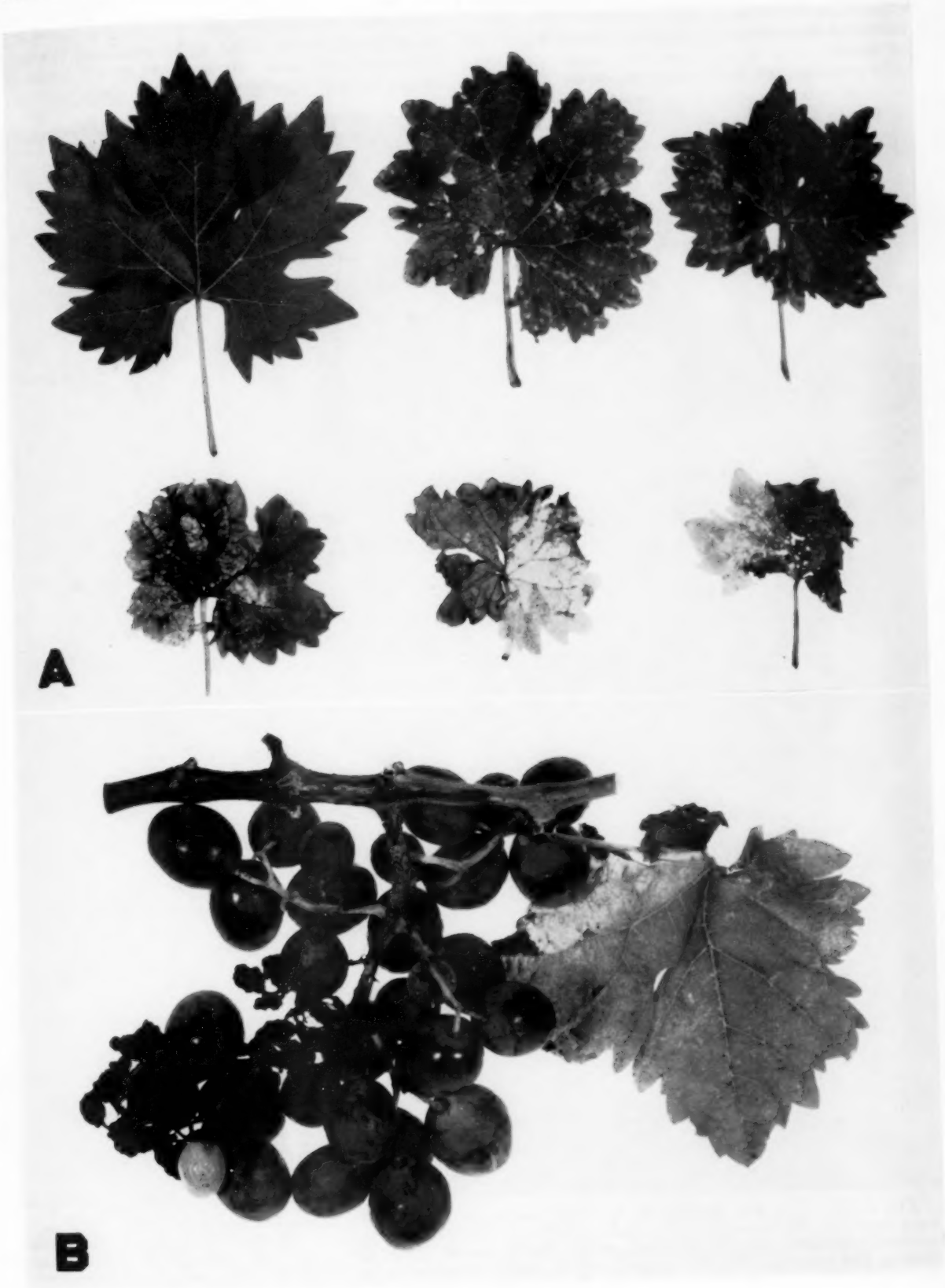
Fig. 1 A) Symptoms of dead-arm on leaves of Tokay grapevines. Upper left leaf healthy, the others showing increasing degrees of spotting, chlorosis, deformity, and tattering. **B)** Grape cluster infected with *Phomopsis viticola*. The cane and cluster stem are almost completely girdled by the corky abrasions resulting from severe fungus attack.


A
B

with string to canes of healthy Tokay vines. These vines also showed disease symptoms after a year in the lathhouse.

At pruning time in the dormant season, 2–3 of the basal buds on a cane are left as a spur for the following season's growth. This is the portion of the cane most heavily infected. Hence, when growth begins in the spring and if moisture is present, these spurs are

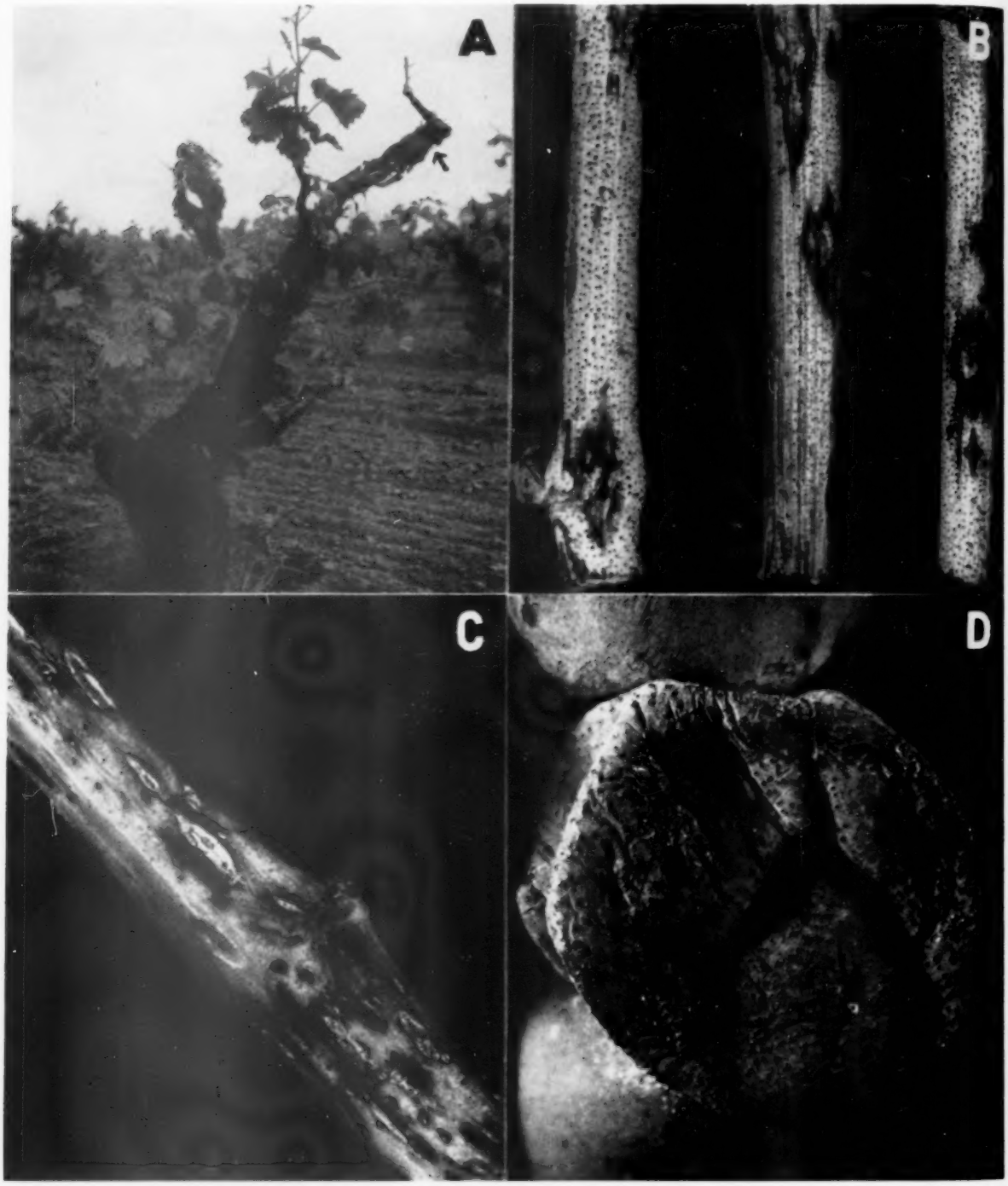


Fig. 2. A) Tokay vine showing the dead-arm condition from which the disease has gained its name. Arrow indicates dead arm. **B)** Segments of grape canes covered with pycnidia of *Phomopsis viticola*. The epidermis has become stained bluish-black, and then bleached, through fungus action. (2×) **C)** Grape shoot infected with the dead-arm fungus. The cortical layers have cracked and the unbroken epidermis has darkened. These areas are surrounded by pycnidia in different stages of development. **D)** Pycnidia covering the surface of an infected grape berry.

in perfect position to provide abundant inoculum for infecting new shoots of the same or adjacent arms of the vine.

For determining the spread of the pathogen within the diseased vine, Tokay canes were collected from vines showing disease symptoms and planted in clay pots with 1 or 2 buds well above the soil level. Water was kept from touching these canes to exclude disease spread by pycnidiospores. After 3 months, 31 of the original 39 buds had produced no growth. These buds were surface-sterilized for 1 minute with mercury bichloride, 1:1000, and tissue was taken from the base of each bud and plated on potato-dextrose agar. The dead-arm fungus was isolated from 23 buds, bacteria or other organisms from 6, and 2 were sterile. Eight of the original buds produced new shoot and leaf growth. Tissue sampling from the new growth indicated that 7 shoots were infected. In 4 cases, petioles were infected, and in one instance the fungus was isolated from a leaf blade. Hence, the fungus may spread slowly within the vine from diseased tissue to new growth, and be disseminated in cuttings and buds.

Pycnidiospores were found to germinate only in water or on culture media that contained high amounts of available water. They did not germinate on dry sterilized grape wood, but germinated abundantly when the wood was moistened with sterile water.

Infection courts.—It has long been recognized that the dead-arm pathogen is a wound invader. Reddick (9) transmitted the disease with pruning tools and through knife wounds. When Coleman (3) inoculated cut shoots, vessels were penetrated 0.75 in. by the fungus in 15 days, and pycnidia formed on the wood in 2 months.

As a test of wound infection on several *V. vinifera* varieties, the shoots of young plants growing in a lathhouse at Davis were aseptically severed, and the cut ends held for 30 seconds beneath the surface of a spore suspension of the pathogen. The cuts were then wrapped in cotton and covered with plastic cloth until callused. The varieties used were Carignane, Green Hungarian, Chardonnay, Muscat Canelli, Ribier, Mataro, Red Malaga, Emperor, Muscat of Alexandria, and Tokay. The cut shoots of control plants were dipped in sterile water. Forty-two days after inoculation, lesions were evident on the stems of the Chardonnay, Emperor, and Muscat of Alexandria varieties. At 60 days, Muscat Canelli and Tokay also showed signs of infection. At one year, all varieties showed definite disease symptoms. By means of tissue platings, *P. viticola* was recovered from all the inoculated plants. Check plants did not produce disease symptoms, and the fungus was not recovered in culture.

The buds and young leaves of grapevines may receive extensive injuries from mites and insects, particularly the Willamette mite, *Tetranychus flavus* Ewing. Such injuries are most pronounced after prolonged periods of rainy or foggy weather, which also correspond with the times when dead-arm symptoms are most severe. The following experiment was designed to test the potential of leaf injury as an infection court, and to determine the amount of moisture necessary to produce extensive fungus penetration. In June, 1956, 36 healthy Tokay and Thompson Seedless cuttings were so placed in a fog chamber that moisture did not fall directly on them, but condensed readily and evenly on their leaves. At various intervals the leaves were pricked or lightly scratched with a hypodermic needle, and a sterile water suspension of *P. viticola* spores was atomized on the wounded areas. The plants were then returned to the fog chamber for different periods, and finally all were removed to a lathhouse. Only a few leaf and stem symptoms were seen during the rest of the 1956 growing season. During May, 1957, these vines were appraised for disease symptoms, and rated: 0 = no symptoms; 1 = mild infection; 2 = severe leaf and cane infection. Table 1 shows the results, together with the periods of moisture before and after inoculation. The evidence indicates that leaf wounds, like those that insects might produce, provided an infection court through which the pathogen penetrated to the petioles and shoots. Furthermore, infection increased with the amount of moisture present, either before or after inoculation.

While still in the bud, grape leaves have a profusion of hairs growing from their lower surfaces. Most of these hairs are broken off as the leaves unfold and expand, which provides numerous wounds for invasion of the host by the pathogen. The openings expose the sub-epidermal tissues. Although the openings are occasionally so large that *P. viticola* spores could literally "drop" into the interior of the young leaf, no instance of direct penetration was observed. Infections of the bud base were found repeatedly on buds artificially inoculated with spore suspensions of the pathogen, with sequence of penetration and infection being the same as that seen in leaf and cane stomates.

Winter killing of immature portions of canes was found to be still another type of wounding that allowed entry of the fungus. As the canes were killed back in the late fall, the pathogen rapidly spread into this area, covering the canes with pycnidia by midwinter. When an unseasonal frost occurred after the start of

Table 1.—*Dead-arm disease symptoms produced on leaves of Tokay vines by spraying spore suspensions of Phomopsis viticola on artificial wounds and submitting the vines to various times in a mist chamber*

Hours in mist		
Preinoculation	Postinoculation	Rating[a]
1	4	0.25
1	1	0.50
4	1	0.50
4	4	0.50
1	8	1.00
8	1	1.50
4	8	1.75
8	8	1.75
8	4	2.00

[a] Based on means of ratings: 0 = no infection; 1 = mild; 2 = severe disease symptoms.

growth in the spring, young shoots were sometimes killed, and the pathogen was found to attack them readily.

However, wounds do not account for all infections of dead-arm in nature. Since stomatas are numerous on the lower surfaces of grape leaves and on canes, this avenue of penetration was examined.

Leaves of healthy Thompson Seedless vines, 0.75–1.50 in. in diameter, were floated on sterile water in Petri dishes with their lower surfaces upward. Drops of a sterile-water spore suspension were placed on the exposed leaf surface and allowed to remain undisturbed for 4 days. At the end of this period, the portions of leaves beneath the inoculum were removed, embedded in paraffin, cut at 8–10 μ on a rotary microtome, and stained with Rawlins' triple stain (8) or hematoxilin plus safranin O (11).

In no instance was penetration of a stoma by fungus hyphae or germ tubes observed, but spore germination on the lower leaf surface was seen repeatedly. Indication that infection had taken place appeared first in the guard cells of the stomata, then in the mesophyll cells lining the sub-stomatal cavity, followed by mesophyll neighboring on the cavity, and lastly in the palisade and epidermal cells of the upper leaf surface directly above the stoma concerned. The protoplasts of attacked cells became disorganized; there was a collection within the cells of material that had the appearance and staining reaction of wound gum; eventually the cells collapsed completely. The fungus mycelium was not stainable in the cells undergoing these changes, but was seen in advance of the reactions. Where stomata were close to vascular bundles, or where several seats of infection overlapped and touched upon bundles, the reactive process could be followed along the vascular system for several millimeters. This condition is readily seen on diseased vines in the field; leaves often show a marked necrosis, which, through leaf expansion, ultimately leads to the tattered effect.

The same sequence of infection was seen at the stomata of green shoots. The fungus moved laterally away from the stoma, generally in the outer cortical tissue, but sometimes directly inward to the cambium and xylem. The host tissues beneath the stoma proliferated, the expanding cell mass ruptured the epidermis, and the reddish-brown sterile bodies were produced. As with leaf stoma infection, the pathogen has not been seen in, or isolated from, the necrotic host tissue, but it has been isolated from the margins of these areas.

Production of these sterile bodies in the development of dead-arm disease has received critical attention by some earlier workers. Reddick mentioned these bodies in his original description of the disease (9), and again in 1914 (10). Coleman (3) observed them during his investigations, but was not able to demonstrate the presence of the fungus in or about them. In the present work, the sterile bodies were first noticed as round, reddish spots on the shoots, surrounded by dark green halos. They became larger, reaching 0.5–1.5 mm in diameter, domed, and dark red, and eventually turned brown. The top later broke away. When many of these bodies occurred close together, they merged to form the corky abrasions characteristic of the disease.

Since leaf infection is most commonly found on the lower 6–8 nodes of diseased shoots, it was felt that leaf age might have a bearing on susceptibility. To explore this possibility, rooted cuttings of several varieties at different stages of growth were placed in a mist chamber for 4 hours, then atomized with a spore suspension of *P. viticola* once every hour for 4 additional hours, and finally allowed to remain in mist for another 24 hours before being returned to the greenhouse bench. All plants were removed to a lathhouse after 3 days. At the end of 30 days, symptom expression was rated as: 0 = no infection; 1 = slight leaf spotting; 2 = mild leaf and/or cane symptoms; 3 = severe disease symptoms. Table 2 shows 1) the stage of shoot development at the time of inoculation, 2) the number of shoot inoculations in each age group, and 3) the whole numbers of shoots falling into the four symptom ratings. The plants used in this experiment were allowed to grow in the lathhouse for a full year following inoculation. At this later time, all the plants showed the disease. It appears evident that leaf age is not of prime importance in susceptibility to infection, but that very young leaves on shoots up to 4 in. long tend to show disease symptoms faster than older leaves, and thus are better indicators of the time and extent of infection.

The most fully expanded leaf on a young shoot 3 in. long is about 2.5 in. wide. The leaf blade on a young shoot seldom lies flat; more often it is folded or cupped around the smaller, younger leaves. Rain water or fog condensation was found to collect between leaves, between leaves and shoot, or in the folds of the bud. As shown previously, if water containing spores of *P. viticola* remained in contact with the leaf surface for only a few hours, infection was accomplished. The leaves in the first 3 in. of growth at the time of infection are those that eventually make up the basal 6–8 nodes, the same leaves that show the greatest amount of leaf spotting, and the same internodes that

TABLE 2.—*A comparison of shoot growth and susceptibility to inoculation by spore suspension of Phomopsis viticola*

Shoot development	No. inoculations	Total in each rating group[a]			
		0[b]	1	2	3
Green buds	11		6	5	
Buds just open	12		5	3	4
1 in.	17	4	4	6	3
2–3 in.	11	2	1	4	4
4–24 in.	16	7	3	6	

[a] Ratings expressed as: 0 = no infection; 1 = slight leaf spotting; 2 = mild leaf and/or cane symptoms; 3 = severe disease symptoms.

[b] Plants rated without symptoms at the end of 30 days all showed symptoms 1 year after inoculation.

show the most severe lesions.

The development of severe infection on the leaves and shoots of newly opened buds was observed through secondary infection in the field during years of exceptionally late spring rains. In 1957, the Tokay-growing district surrounding Lodi, San Joaquin County, received several days of rainy and cloudy weather when the buds were just opening. Dead-arm infection on the lower internodes of new shoots was severe in vineyards where control programs were not in force. Then, when canes were about 18 in. long, the area again had strong rains, which continued over 6 days. Three weeks later, when another 6 internodes of growth had developed, they were covered with young pycnidia and incipient lesions. Hence, 2 areas showing disease symptoms were to be found on the same canes, one at the base and one about 2 ft. higher. In both cases, the leaves that had been close to the growing tip of the shoot at the time of rainfall were the ones that showed disease symptoms.

Disease development.—In California, as elsewhere, the incubation period for the dead-arm pathogen in the host is about 30 days (3, 4, 10). Fungus hyphae, once infection is established, move intracellularly through any of the tissues of leaf, petiole, tendril, cluster stem, or cane. Passage from healthy cell to cell is through the pit areas, as shown by Coleman (3). Little or no effect is evident in the xylem of infected shoots, although the fungus is to be found in this area. The phloem becomes increasingly disorganized as the disease progresses. The narrowness of the cambium and lack of lignification, also noted by Coleman in American varieties, was observed repeatedly in *V. vinifera*. Whereas the unaffected side of a cane shows the regular formation of phloem fiber bundles, diseased portions have practically no fibers, or they are formed singly and seated in parenchyma of atypical arrangement. The cambium of diseased canes is often wavy, and the cells being cut off on either side are smaller and thinner-walled than normal.

The reduction in starch content of invaded cells, mentioned by Coleman, was not observed in California varieties, except in those tissues already becoming necrotic, e.g., margins of leaf and cane spots. However, a "yellowish-brown substance," observed by Coleman, "extremely resistant to solvents and chemicals in general, which has the characteristics of wound gum," was often seen in sectioned material. Since the pathogen produces large quantities of oil (7), it is believed that these deposits in host cells, almost always close to the borders of lesions, are metabolic by-products of fungus growth.

The distortion of the cambium and cortex is always found beneath the corky abrasions on the surface of infected canes. When these corky areas extend around the shoot, the diseased cortex and cambium are likewise affected, resulting in a girdling of the cane. When such canes are left as spurs for the following year's growth, they are destroyed by the fungus, and the dead-spur symptom is evident. When the girdling occurs further down the arm, the characteristic dead-arm condition is obtained.

Pycnidia develop from hyphal mats growing through the subepidermal portions of canes. They usually become well developed before breaking through the epidermis. In berries, as previously shown by Gregory (4), pycnidia are seated in the hypodermal parenchyma, usually over lenticels.

Varietal susceptibility.—In 1928, Coleman, writing about the American grape varieties, said that he had yet to see a variety free of the disease (3). Anderson and Colby (1) catalogued 56 varieties of American grapes, none of which were resistant. The same holds true for the European varieties grown in California. In the northern portion of the San Joaquin Valley, the following varieties are commonly grown, and the dead-arm fungus was isolated from all of them: Tokay, Olivette blanche, Cornichon, Almaria, Palomino, Zinfandel, Black Prince, Thompson Seedless, Cardinal, Mission, Valdepenas, Emperor, and Alicante Bouschet. Most of these varieties are grown on St. George rootstocks (*V. rupestris* var.), a variety also found infected in the field. Artificial inoculations produced the disease on Carignane, Green Hungarian, Chardonnay, Mataro, Muscat Canelli, Ribier, Red Malaga, and Muscat of Alexandria. No attempts have been made to record the individual degrees of susceptibility qualitatively.

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TRANSMISSION OF STRAINS OF BARLEY YELLOW DWARF VIRUS BY TWO APHID SPECIES[1]

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SUMMARY

During a 2-year period, 80 New York oat plants with symptoms of disease caused by barley yellow dwarf virus (BYDV) were tested for presence of the virus by use of apple grain (AG) aphids (*Rhopalosiphum fitchii*) and English grain (EG) aphids (*Macrosiphum granarium*). Virus was transmitted only by EG aphids from 67 of the samples, only by AG aphids from 2 samples, and by both aphids from 6 samples. Two AG-isolates and 2 EG-isolates, initially transmitted only by AG and EG aphids, respectively, were selected for further study. All 4 isolates caused symptoms typical of infection by BYDV in several hosts, were persistent in their aphid vectors, and had other biological characteristics of BYDV as determined by comparisons with a known strain of the virus.

Vector specificity of the 4 isolates remained essentially unchanged after a total of 129 transfers that included 11 serial transmissions of each isolate. In all cases, however, occasional transmission by the "nonvector" aphid occurred; such transmissions of AG-isolates by EG aphids were more common than were transmissions of EG-isolates by AG aphids. Even after AG aphids had been reared on plants infected with either EG-isolate, they transmitted virus to only 3 of 173 plants. EG aphids reared on plants infected with an AG-isolate sometimes effected transmission to a high percentage of the test plants. The observed vector specificity of both kinds of isolates is considered to be relative and not absolute, since the virus occasionally transmitted by a "nonvector" appeared to be the specific isolate introduced into the source plant and not a mutant or other selection of virus from it.

Although the AG-transmitted isolates are considered to be similar to strains of BYDV reported from other areas, the EG-transmitted isolates seem to represent a strain of the virus common in New York but different from those that are common in other areas of the United States where studies have been made.

Several aphid species are known to be vectors of barley yellow dwarf virus (BYDV) in the United States, but the apple grain (AG) aphid (*Rhopalosiphum fitchii* Sand.), generally considered the best vector, has usually been used in studies with BYDV (1, 2, 6, 7, 11, 13). Information is now available, however, on what appear to be strains of BYDV that are not regularly transmitted by the AG aphid. One such strain, transmitted by the English grain (EG) aphid (*Macrosiphum granarium* Kirby) but not by the AG aphid, was obtained from 62 field samples collected in Washington during a 2-year period by Bruehl (4) and Toko and Bruehl (14, 15). Similar strains have been frequently encountered in New York. A total of 80 oat plants with symptoms of infection by BYDV were collected in New York fields and tested in this laboratory in 1957 (9) and 1958. Virus was transmitted from 67 of these samples by EG aphids only, from 2 samples by AG aphids only, and from 6 samples by both aphids. Cooperative tests with G. W. Bruehl have shown that the basis for these differences in aphid transmission of BYDV rests with the virus and not with peculiarities of the aphids used in New York and in Washington (4, 10).

The extent and nature of this apparent vector specificity of the New York isolates of BYDV are the subject of this paper.

MATERIALS AND METHODS.—All stock colonies of virus-free AG[3] and EG aphids were maintained on caged oat (*Avena sativa* L.) or barley (*Hordeum vulgare* L.) plants. The 4 precautions taken to keep these colonies free of virus were as follows: 1) they were grown on plants in a laboratory away from all plants infected by BYDV; 2) each colony was started from 10–20 newly emerged nymphs placed on young cereal plants; 3) the cage for each colony was opened only when the aphids were used; and 4) the entire colony was discarded immediately after use. Although all aphids maintained in this way have remained virus free, some aphids from each colony used always were tested as controls.

Four New York isolates were used regularly in these studies. Each isolate was originally transmitted from a single oat leaf collected in the field and tested with both AG and EG aphids (9). Two of the isolates (EGV-1, EGV-2) were originally transmitted only by EG aphids, and 2 isolates (AGV-1, AGV-2) were originally transmitted only by AG aphids. Isolate No. 1 of each series came from a field in Broome County; isolate No. 2 of each series came from Caldwell Field, on the Cornell University Farm. A fifth isolate (EGV-3), used in 1 test, was similar to isolate EGV-2. Unless stated otherwise, transmission of each isolate was by means of the aphid that had effected the original transfer. A sixth isolate (Ill-1), a moderately severe strain of BYDV supplied by R. M. Endo from Illinois in 1956, was used as a reference strain of the virus in parallel tests with the New York isolates. This reference strain was usually transmitted by means of AG aphids.

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[3] Several entomologists identified this species as *R. fitchii* (AG), but one worker identified it as *R. padi*.

Acquisition feedings of 24–48 hours were usually on detached leaves or half-leaves, as described previously (9), except that plastic dishes with tight-fitting covers were used instead of Petri dishes in most tests. When detached half-leaves were used, 1 half of the leaf was infested with nonviruliferous AG aphids and the opposite half-leaf with nonviruliferous EG aphids. In the latest tests, the infested leaves were incubated at 15°C during this acquisition feeding, but in most of the experiments described they were stored at room temperature. When acquisition feeding periods of more than 48 hours were desired, aphids were caged on intact source plants in the greenhouse. Unless stated otherwise, inoculation test feedings were for 3 days on caged plants in the greenhouse. At the end of this inoculation test feeding period, the aphids were killed by fumigation with lindane in a closed chamber. The plants were then placed on a greenhouse bench under supplemental illumination, where they were observed for at least 4 weeks.

In most cases, Coast Black oats (C.I. 1025) or California Red oats (C.I. 1026) were used as test plants. Atlas 46 barley (C.I. 7323) was used sometimes. ("C.I." refers to accession number of Cereal Crops Research Branch, Crops Research Division.) At the end of the acquisition feeding period, aphids were transferred by means of a camel's-hair brush to seedlings of these plants in groups of about 10 aphids per plant. Most of the aphids in each group were late-instar apterous females, but other forms were often included in each group.

RESULTS.—*General biological properties of New York isolates.*—Symptoms caused by each of the 4 New York isolates, in the field plants from which they were obtained as well as in greenhouse-grown oats and barley, were typical of infection by BYDV as described by others (1, 8, 12). Although oats infected by isolates EGV-1 and EGV-2 frequently developed symptoms a few days before oats infected by the AG-transmitted isolates, the disease caused by each of the isolates did not differ consistently in severity. Symptoms in the greenhouse generally first appeared about 2 weeks after the start of the inoculation test feeding period.

Oats, barley, and wheat (*Triticum aestivum* L.) were consistently susceptible to all virus isolates. Nine grasses, seeds of which had been furnished by G. W. Bruehl, were tested for susceptibility to isolates AGV-2 and EGV-2 and to Ill-1, the reference strain. Tests were based on recovery of virus from plants on which about 10 viruliferous aphids had fed for at least 3 days. *Bromus mollis* L. and *Lolium perenne* L. were susceptible to all 3 isolates. None of the isolates were recovered from inoculated plants of *Bromus catharticus* Vahl. Plants of *Phleum pratense* L., *Poa ampla* Merr., and a dwarf selection of *Dactylis glomerata* L. were susceptible to AGV-2 and Ill-1, but not to EGV-2. *Agropyron cristatum* (L.) Gaertn., in contrast, was susceptible only to EGV-2. *Bromus inermis* Leyss. was susceptible only to Ill-1, whereas *Agropyron intermedium* (Host) Beauv. was susceptible to all isolates except the Illinois strain. In 5 of the above cases, however, virus was recovered from only 1 or 2 of the 3 or 4 plants of each species tested with each virus isolate. Genetic variation among individual grass plants and variability in aphid feeding on different hosts may be as important as variations among virus isolates. At least, these results agree with the general variability in host range of strains of BYDV reported by Bruehl and Toko (5) and emphasize the danger in generalizing about the host range of BYDV.

Since persistence in its aphid vector is a characteristic of BYDV, persistence of each of 2 New York isolates (AGV-2 and EGV-2) was compared with that of the Illinois reference strain. In 1 series of tests, nonviruliferous aphids were caged on infected plants for acquisition feedings of 1 day. Individual aphids

TABLE 1.—*Transmission of 3 isolates of barley yellow dwarf virus by single apple grain (AG) or English grain (EG) aphids after 1 day of acquisition feeding followed by 1 day of inoculation test feeding on successive single oat plants*[a]

Virus isolate[b]	Vector	Infections following each of 21 successive serial transfers to single oat plants																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
EGV-2	EG	+	+	+	+	+	+	+	+	+	+	+	D									
		+	+	+	+	+	+	+	+	—	+	+	+	D								
		+	+	+	+	+	+	+	+	+	+	+	D									
		+	+	+	+	+	+	+	+	+	+	D										
		—	+	+	+	+	+	+	+	+	+	D										
AGV-2	AG	+	+	+	+	+	+	+	—	+	—	—	+	—	+	+	+	+	—	+	—	—
		+	+	+	+	+	+	+	+	+	—	—	+	+	+	+	+	—	—	+	—	+
		+	+	—	+	+	—	+	+	—	+	+	+	+	+	+	+	—	—	—	D	
		+	+	+	+	+	+	+	+	+	+	+	—	—	+	D						
		+	+	+	+	—	+	—	+	+	—	+	+	+	+	+	+	+	+	—	—	+
Ill-1	AG	—	—	—	+	+	+	+	—	+	—	+	—	—	+	+	—	+	+	+	—	+
		—	+	+	+	+	+	—	+	—	+	+	+	+	+	—	D					
		+	+	—	+	+	+	+	+	+	—	—	+	+	+	+	+	+	+	+	+	D
		+	+	+	+	—	—	+	+	+	—	+	—	+	—	—	+	—	—	—	+	+
		+	+	—	+	+	+	+	+	+	+	—	+	+	—	—	+	+	+	—	—	D

[a] + indicates that the plant became infected; — indicates that it did not become infected; D indicates that the aphid died.

[b] Isolates EGV-2 and AGV-2, obtained from New York, are regularly transmitted only by EG and AG aphids, respectively. Ill-1 is a reference strain of BYDV from Illinois.

TABLE 2.—*Number of infections during 11 serial transmissions of 4 New York isolates of barley yellow dwarf virus by apple grain (AG) and English grain (EG) aphids after acquisition feeding on opposite halves of detached leaves*

Virus isolate[a]	No. successful transmissions by each aphid[b]		No. test plants infected following feeding by each aphid[c]	
	AG	EG	AG	EG
EGV-1	3/34	34/34	3/110	121/125
EGV-2	1/29	29/29	4/104	115/115
AGV-1	36/36	6/36	123/128	14/120
AGV-2	30/30	9/30	111/111	14/101

[a] EGV and AGV isolates were originally transmitted only by EG and AG aphids, respectively.
[b] Numerator is number of tests in which at least 1 plant became infected; denominator is total number of tests completed.
[c] Numerator is number of oat test seedlings that became infected; denominator is number of seedlings that were infested with about 10 aphids in a 3-day inoculation test feeding period following acquisition feedings of 24-48 hours.

were then transferred to single California Red oat seedlings. Each aphid was transferred to a new oat seedling each day thereafter until the aphid died or the test was ended. Data for 5 aphids that lived the longest in each group are in Table 1. Since single aphids were used, these data are unequivocal evidence for the persistence of each of the isolates in its aphid vector. EG aphids transmitted isolate EGV-2 as long as they lived—12 days in 1 case. Individual AG aphids retained isolate AGV-2 or Ill-1 for as long as 21 days.

Results of similar tests, in which 3-day acquisition feedings were used, were similar to those reported in Table 1, except that fewer plants escaped infection near the end of the tests than when 1-day acquisitions were used.

In similar tests, with 1-hour acquisition feedings, only an occasional plant became infected.

Oat plants infected with isolate AGV-2 or isolate EGV-2 were not protected from infection by the other isolate. Similar lack of cross-protection between strains of BYDV has been observed by Allen (1) and Toko and Bruehl (15).

Vector specificity of the New York isolates.—Each of the New York isolates had been originally transmitted from the field sample either by AG or EG aphids, but not by both. Subsequent transmission of the 4 isolates by both aphids has been tested over a period of 18 months in a total of 129 transfers made by use of detached half-leaves (9). The original vector specificity of each isolate has remained essentially unchanged during these transfers, which included 11 serial transmissions of each isolate (Table 2). Transmission of each isolate by the "nonvector" aphid occurred at least once. Occasional transmission of isolate AGV-1 or AGV-2 by EG aphids was more common than was transmission of isolate EGV-1 or EGV-2 by AG aphids (Table 2).

In this type of test (Table 2), acquisition feedings were usually 24 hours, and never more than 48. The importance of longer acquisition feedings in this occasional transmission by the "nonvector" was tested by rearing aphids on infected source plants. Nonviruliferous AG aphids were placed on oats infected with isolate EGV-1 or EGV-2; EG aphids were placed on plants infected by isolate AGV-1 or AGV-2. After several weeks, during which time colonies developed, aphids were transferred to oat test seedlings (10 aphids per plant) for a 3-day inoculation test feeding. Comparative transmissibility of the 4 isolates was determined in 6 experiments of this type. In most tests, there was a striking lack of transmission of the EG-isolates by AG aphids; vector specificity was also apparent in the tests with the AG-isolates, but it was less marked than that of the EG-isolates (Table 3). In all 6 tests with AGV-2, for instance, EG aphids effected some transmission; in 3 of the 6 tests, most of the test plants became infected. Although EG aphids transmitted isolate AGV-1 in 3 of 6 tests, the number of transmissions was lower than for AGV-2.

Results of both types of tests (Tables 2 and 3) indicate that the vector specificity of the EG-isolates is greater than that of the AG-isolates. Results of parallel tests with the Illinois reference strain have generally been similar to those with New York isolates AGV-1 and AGV-2, not only with detached half-leaves but also when aphids were reared on source plants.

Occasional transmission of the New York isolates by the "nonvector" provides an approach for investigating the type of specificity involved. As has been pointed out previously (9), there are at least 2 general interpretations of these data. First, specificity may be absolute; occasional transmission by the "nonvector" may involve selection of a mutant or of a different virus strain from a mixture and may not represent actual transmission of the predominating strain present. Second, specificity may be relative; the occasional transmission of each isolate by the "nonvector" may be merely a reflection of inefficient transmission of that isolate.

These 2 possibilities were investigated in tests with

TABLE 3. *Transmission of 4 isolates of barley yellow dwarf virus by apple grain (AG) or English grain (EG) aphids reared on virus-infected source plants*

Test No.	No. of plants infected in tests with aphids reared on plants infected with virus isolate as indicated[a]			
	AG aphids reared on EGV-1	AG aphids reared on EGV-2	EG aphids reared on AGV-1	EG aphids reared on AGV-2
1	0/12	0/12	1/3	10/12
2	0/7	0/5	0/11	1/12
3	0/15	0/18	2/21	5/15
4	0/12	1/12	0/12	2/12
5	2/20	0/20	3/20	20/20
6	0/20	0/20	0/20	16/20
Total	2/86	1/87	6/87	54/91

[a] Numerator is number of oat test seedlings that became infected; denominator is number of seedlings that were infested with about 10 aphids each for a 3-day inoculation test feeding. EGV and AGV isolates were originally transmitted only by EG and AG aphids, respectively.

TABLE 4.—*Transmission of 4 isolates of barley yellow dwarf virus following an occasional transmission of an isolate by "nonvector" aphids reared on source plants*[a]

Virus Isolate	Transmitted by aphid indicated	No. cases tested	No. of test plants infected following feeding on opposite halves of detached leaves by aphid indicated[b]	
			AG	EG
AGV-1	EG	8	23/24	1/24
AGV-2	EG	16	52/52	4/53
EGV-1	AG	2	0/6	6/6
EGV-2	AG	1	0/3	3/3
EGV-3	AG	2	0/6	6/6

[a] AGV isolates were regularly transmitted by apple grain (AG) aphids and occasionally by English grain (EG) aphids. EGV isolates were regularly transmitted by EG aphids and occasionally by AG aphids. The occasional transmitter is termed a "nonvector."

[b] Numerator is number of oat test seedlings that became infected; denominator is number of seedlings that were infested with about 10 AG or EG aphids each day for a 3-day inoculation test feeding after acquisition feedings of 24-48 hours.

some of the plants that occasionally became infected in tests with "nonvector" aphids. The ability of AG and EG aphids to transmit virus from leaves of such plants was tested by means of the detached-half-leaf method. Most of the tests were made with plants to which virus had been transmitted by "nonvector" aphids reared on source plants (Table 3), but the results of similar tests with plants from the serial transfers (Table 2) were generally in agreement. In all of the 29 tests made, the transmission pattern of an isolate involved in an occasional transmission by a "nonvector" proved, in subsequent transmission tests with both aphids, to be the same as that of the isolate after transmission by its regular vector (Table 4). In other words, no selection of virus strains appears to be involved in the occasional transmission of either type of isolate by a "nonvector" aphid. Thus, vector specificity of BYDV is relative, not absolute.

DISCUSSION.—In some ways, the relationship of strains of BYDV to AG and EG aphids is analogous to the relationship of 2 strains of potato yellow dwarf virus to 2 species of leafhoppers. Black (3) found that the New Jersey strain of potato yellow dwarf virus was transmitted by 1 leafhopper species and that the New York strain was transmitted by another; neither species transmitted both virus strains. This vector specificity of strains of potato yellow dwarf virus appears to differ from that of BYDV, however, for the former was reported to be absolute, whereas the specificity of the New York isolates of BYDV is relative. It should also be kept in mind that Black clearly established the relationship of the strains of potato yellow dwarf virus, whereas the relationship of the strains of BYDV rests solely on similarities of some of their biological properties.

In most respects the New York isolates of BYDV appear to be similar to the vector-specific strains studied in Washington (14, 15). The main difference is that the specificity of the Washington strains was absolute, whereas that of the strains studied in New York was relative. This difference may be more a reflection of the kinds of tests that were emphasized in each study than of real differences between the vector-specific strains of the 2 regions. In Washington, for example, the 6 attempts to transmit each strain by means of the "nonvector" were based on acquisition feedings of 36 hours. Studies with the New York isolates suggest that an occasional transmission by the "nonvector" would be less likely under those conditions than if longer acquisition feeding periods had been used.

The vector specificity of BYDV appears to be sufficient to be of possible practical importance. It shows the danger of using only 1 aphid species to confirm the presence of the disease in areas where the predominating virus strain is unknown. Since at least 5 aphid species (8, 12) are vectors of the virus in the United States, the occurrence of additional vector-specific strains of BYDV is a good possibility. For example, virus was recovered from all but 5 of the 80 New York field plants that were tested with AG and EG aphids in this study. One of many possible explanations for failure to recover virus from 5 samples is that they were infected with another vector-specific strain, transmissible only by an aphid different from the 2 used. In nature the occurrence of vector-specific strains of BYDV is probably a function of the predominating aphid in each area, or of seasonal fluctuations of different aphids with respect to development of the cereal crops, to development of other hosts, and to the time during the season when samples are collected.

The New York isolates of BYDV that are regularly transmitted only by EG aphids (EGV-1, EGV-2) seem to represent a strain of the virus that is common in New York (9) but different from strains that are common in other areas of the United States where studies have been made (1, 4, 8, 12, 15). Although a similar strain has been reported from Washington, EG-specific isolates of the virus are apparently rare in that area (4, 15). The New York isolates that are regularly transmitted only by AG aphids (AGV-1, AGV-2) are probably of the same general type as those commonly found in other areas where BYDV has been found to be transmitted less efficiently by EG aphids than by AG aphids (13). Since vector specificity of the AG-isolates is less pronounced than that of the New York EG-isolates, it is not surprising that vector specificity of BYDV was not encountered in the early studies in other areas. It is possible that there exists a series of AG-transmitted strains that differ in how readily they are transmitted by EG aphids. At one end of such a series would be a strain never transmitted by EG aphids, such as was reported from Washington (15). Next in the series might be a strain similar to the New York isolate (AGV-1) that was transmitted only occasionally by EG aphids. The other New York isolate (AGV-2), which was sometimes transmitted to a high percentage of the test plants by EG aphids,

might be next in line to still other strains that would be even more readily transmitted by EG aphids.

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BIOLOGICAL PROPERTIES OF RED CLOVER VEIN MOSAIC VIRUS[1]

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SUMMARY

Lotus corniculatus, *Nicotiana repanda*, and *Capsicum frutescens* were found to be insusceptible to red clover vein mosaic virus (RCVMV); *N. tabacum* was found to be susceptible in certain instances. The virus did not become systemic in the local-lesion host, *Gomphrena globosa*. Mechanical transmission was most successful in all hosts when young leaves were inoculated. Infected red clover plants proved better source plants for the virus than did infected plants of *Pisum sativum*, *G. globosa*, or *Vicia faba*.

As a diluent, cysteine solutions at pH 4 or 5 were superior to cysteine solutions at pH 6, to acetate or phosphate buffers at pH 4, 5, or 6, and to deionized water; water was superior to acetate or phosphate buffers. Clarifying the inoculum by centrifugation before freezing increased infectivity. Plants of *G. globosa* darkened for 24 hours after inoculation produced more lesions than did the control plants kept in continuous light. Darkening *G. globosa* for 24 hours prior to inoculation proved detrimental to lesion formation.

Apparent virus titer was highest in January and lowest in May. There was no seed transmission of the virus in *P. sativum*, but a high percentage occurred in *V. faba*. Amorphous and crystalline inclusion bodies were found in leaf hairs of diseased red clover. In greenhouse tests, infection of red clover by RCVMV decreased vigor and longevity, decreased dry weight, and increased heat susceptibility. RCVMV infection decreased the rate of apparent photosynthesis of red clover leaves; this depression increased as symptom severity increased. Virus infection increased the rate of respiration; this effect was most pronounced 3 weeks after inoculation and coincided with the onset of symptom development.

The disease caused by red clover vein mosaic virus (RCVMV), *Marmor trifolii* Holmes, was first described in 1937, by Osborne, (17). Twelve years later, Hagedorn and Walker (9) reported a disease of canning peas (*Pisum sativum* L.) in Wisconsin caused by Wisconsin pea stunt virus, considered to be identical with RCVMV (6, 7). The literature on this virus and the disease it causes has been reviewed (14, 24).

Various cultivated and wild leguminous plants serve as reservoirs for RCVMV (3, 4, 5, 8, 11, 21). The virus is transmitted to some extent thorugh the seed of red clover (16), by 2 species of aphids (4, 5, 17, 18, 24), and by mechanical methods (8, 24). Insect transmission may account for the widespread endemic incidence of the disease. The virus incites vein mosaic of red and other clovers, collapse of crimson clover (*Trifolium incarnatum* L.), and stunting of field and

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garden peas and broad beans (*Vicia faba* L.) (24). The economic importance of RCVMV appears to be increasing from year to year. It causes severe losses in the Wisconsin canning pea areas (9), reduces longevity in red clover stands by diminishing its winter hardiness, increases the susceptibility of red clover to root rot (19), and reduces yielding ability in general (16, 22).

The recent discovery of *Gomphrena globosa* L. as a local-lesion host for RCVMV (20) has provided a valuable tool for quantitative work with this virus. This discovery led to the present investigation, the main objectives of which were to develop methods of using this plant in assay work with RCVMV and to use such methods in determining some of the biological and physical properties of this virus. In addition, investigations were made of the effect of this virus on plant vigor and longevity, on photosynthesis, and on respiration.

MATERIALS AND METHODS.—*Virus and plants.*—The strain of RCVMV used was originally obtained from a naturally infected plant. It was passed 5 times through local lesions on *G. globosa*, and finally transmitted to a healthy red clover plant. This plant was cloned when it showed symptoms, and the resulting plants served as sources of inoculum. The identity of the strain was substantiated on the basis of host range and physical properties, and also by a direct comparison of the strain with Wisconsin pea stunt virus.[3]

All plants grew under ordinary greenhouse conditions at a temperature of about 21°C (temperatures above 28° mask symptoms, and temperatures below 16° delay symptom development (7)).

From early May to early September, shade was provided by whitewash on the greenhouse roof. From November to April, artificial illumination lengthened the day to 17 hours and was added to daylight during cloudy days.

In the majority of experiments, plants were grown from seed in 2- to 4-in. clay pots in sterilized soil. Clones were obtained by cutting nodes from healthy or diseased clover plants, rooting them in vermiculite in a mist chamber, and transferring them after 3 weeks to 4-in. pots containing sterilized soil.

Throughout this investigation, *G. globosa* was used for quantitative assays, most of which were made according to an incomplete block scheme (23); data from such tests were analyzed statistically by the Student-Neumann-Keuls test (25). Also, a 2-solution scheme for direct comparison of 2 treatments was employed; results of such tests were statistically evaluated by analysis of variance. Lesions were counted about 10 days after inoculation. All experiments were repeated at least once.

Method of inoculation.—The virus was transmitted mechanically in all experiments. Inoculum was usually prepared from about 3-week-old red clover leaves showing pronounced symptoms. Inoculated leaves of *G. globosa* were used as inoculum in some experiments; virus was extracted either from single lesions or from whole leaves with many lesions. When the inoculum was prepared from species of other plants, rapidly expanding leaves were used.

Deionized water was used as diluent in most tests, although buffers were used in some. The proportion of plant tissue to diluent varied from 1:5 to 1:50 by weight. After leaves were detached from the source plant, they were either placed immediately in the diluent and ground with a pestle in a mortar or frozen 24 hours at −10°C and then placed in the diluent and ground to a pulp. The fibrous material was filtered off through fine-mesh cheesecloth, and the resulting juice was rubbed onto leaves of test plants with a cheesecloth pad. Four strokes were used on leaves of small-leaved species and 8 strokes on leaves of *G. globosa* and *Nicotiana* species. After every 4 strokes, the pad was saturated anew. Each inoculum was tested on *G. globosa*.

Before inoculation, test plants were dusted with 400-mesh Carborundum by means of a powder blower. After inoculation, they were rinsed and replaced on the greenhouse bench.

Inoculations were made in the evenings after 8 p.m. unless otherwise indicated. The success of transmission was detected either by symptom development or by back-tests to *G. globosa*.

RESULTS AND DISCUSSION.—*Biological studies.*—1) *Host range.*—In host-range studies, young seedlings or young leaves of axillary shoots of old plants were inoculated with a 1:5 aqueous dilution of fresh red clover leaf tissue. The age of the plant at the time of inoculation had no apparent effect on transmission to clovers or to *V. faba* as long as the virus was introduced into young growing leaves.

The species listed in the literature as susceptible to RCVMV (14, 24) that were found to be susceptible to the strain used in the present work were *Trifolium pratense* L., *T. incarnatum* L., *T. hybridum* L., *T. repens* L., *Pisum sativum*, *P. sativum* L. var. *arvense* Pior., *Vicia faba*, and *Lathyrus odoratus* L. *G. globosa* did not become systemically infected by RCVMV, but reacted only with the formation of local lesions like those previously described (20). In several tests made at different times of the year, virus was never recovered from uninoculated leaves that had developed after inoculation of other leaves on the same plant. *T. pratense*, *T. incarnatum*, *P. sativum*, *V. faba*, and *G. globosa* reacted to Wisconsin pea stunt virus in the same way as they did to RCVMV.

The present results depart from results reported in the literature mainly concerning the susceptibility of *Nicotiana tabacum* L. Several authors (7, 10, 17) clearly describe this species as insusceptible, but serial transfers from the tobacco plants used in the present study demonstrated that this is not always the case. Twelve inoculated tobacco plants were assayed by inoculations to *G. globosa* from inoculated leaves

[3] Kindly provided by D. J. Hagedorn, Departments of Agronomy and Plant Pathology, University of Wisconsin, Madison.

and from uninoculated leaves from middle and top parts of plants 32 and 54 days after inoculation. Also, 1:5 dilutions from leaves that had been inoculated 32 days earlier were rubbed onto healthy tobacco leaves. This procedure was repeated twice before a final transfer back to red clover, in which typical veinclearing developed.

Bioassays on *G. globosa* indicated that the virus moves slowly and does not reach a high concentration in *N. tabacum*. In inoculated leaves, virus titer was higher after 32 days than after 53 days, but virus was detectable in top and middle leaves only after 53 days. Thus, the data clearly indicate that RCVMV can multiply to a limited extent in tobacco, although no symptoms are induced.

The tests were made in midwinter, as was a repetition that gave similar results. In tests made during the summer, no evidence was obtained of infection in tobacco.

Species reported as insusceptible in the literature, and also found insusceptible in the present study, were *Solanum tuberosum* L., *Nicotiana glutinosa* L., *N. sylvestris* Spegaz. & Comes (17), *Lupinus angustifolium* L. (21), *Cucumis sativus* L., and *Lycopersicum esculentum* Mill. (7). In addition, *Nicotiana repanda* L., *Capsicum frutescens* L., and *Lotus corniculatus* L. were found insusceptible; these 3 species have not previously been reported as insusceptible.

2) *Seed transmission.*—Thirty plants of *P. sativum* were successfully inoculated with a 1:10 aqueous dilution of infected leaf tissue. Seeds from infected plants were sown and the seedlings assayed for RCVMV; no indication was obtained of virus transmission through seed. In similar tests with *V. faba*, however, evidence of seed transmission was obtained. Eighteen seeds from 6 infected plants were sown. Only 3 seedlings grew, and these were found to be infected. Only 3 seeds were obtained from these plants; these were planted, and one seedling was produced. It proved to be infected, and its seed also produced an infected plant. The tests were continued for 3 more generations, with one infected seedling resulting from one seed in each generation. Thus, the virus was passed through 6 generations.

3) *Source plants.*—Preliminary assays indicated that different source plants differed in virus titer and that the best sources were *T. pratense*, *P. sativum*, *V. faba*, and *G. globosa*; consequently, these 4 were compared as source plants. Inoculum (1:5 aqueous dilutions of fresh tissue) from each of these was applied to *G. globosa* according to the incomplete block design. In one experiment, the numbers of lesions (on 24 test leaves) induced by the extracts from the various source species were as follows: *T. pratense*, 2471; *P. sativum*, 923; *G. globosa*, 834; and *V. faba*, 34. When the experiment was repeated several weeks later, the respective lesion counts were 241, 134, 67, and 42. Statistical analysis of the combined results indicated that *T. pratense* is the best source plant and *V. faba* the poorest, the difference being statistically significant at the 1% level.

4) *Diluents.*—Since the biological activity of some viruses appears to be increased by certain diluents, such as antioxidants (13) and phosphate buffers (12, 27), such materials were compared with deionized water as diluents for RCVMV. The antioxidant tested was cysteine hydrochloride at 0.1*M* concentration and brought to pH 4, 5, 6, and 7 with *M* NaOH solution. To determine if the pH or the compound affects virus activity, cysteine was not only compared with water but also with 0.1*M* phosphate and acetate buffers of pH 4, 5, and 6.

The cysteine solutions were always used shortly after preparation, since cysteine is very unstable. For the comparison, comparable fresh infected clover leaves were ground in the presence of a standard amount of diluent (1:5). Comparisons were made on *G. globosa*.

At pH 4, inocula diluted with phosphate buffer or with water (pH 5.3) induced comparable numbers of lesions (Table 1); cysteine was superior to either of these. Acetate was phytotoxic, and inocula diluted with it induced no lesions; however, so few lesions were induced by inocula diluted with water or with phosphate buffer that the difference between any 2 of these 3 inocula was not statistically significant.

At pH 5, the lesion counts when water and phosphate buffer were used did not differ; again, no lesions resulted when acetate buffer was used, and cysteine was superior to the other 3 treatments (Table 1). Acetate was again phytotoxic, although less so than at pH 4.

At pH 6, inocula diluted with water, phosphate buffer, and acetate buffer yielded similar lesion counts (Table 1); again, cysteine was superior to the 3 other diluents. At pH 7, only water and cysteine were

TABLE 1.—*Relative infectivities of 1:5 extracts of infected (red clover vein mosaic virus) clover leaves prepared with various diluents and tested on Gomphrena globosa*

Diluent	pH	Total no. of lesions[a] Assay I	Assay II	Mean no. of lesions/leaf[b]
Water	5.3	15	10	1.0
0.1*M* cysteine	4.0	169	159	13.7**
0.1*M* phosphate buffer	4.0	6	5	0.5
0.1*M* acetate buffer	4.0	0	0	0.0
Water	5.3	18	17	1.5
0.1*M* cysteine	5.0	159	200	15.0**
0.1*M* phosphate buffer	5.0	4	9	0.5
0.1*M* acetate buffer	5.0	0	0	0.0
Water	5.3	27	10	1.5
0.1*M* cysteine	6.0	113	98	8.8**
0.1*M* phosphate buffer	6.0	16	9	1.0
0.1*M* acetate buffer	6.0	1	1	0.0
Water	5.3	243	269	22.4**
0.1*M* cysteine	7.0	106	116	9.3

[a] Each inoculum applied to 24 leaves. In the first 3 tests an incomplete block design was used. In the 4th test, made at a different time of the year, inocula were compared on opposite leaves.

[b] Double asterisk indicates that difference between the indicated mean and any other mean in the same test is statistically significant at the 1% level.

compared; the results were in favor of water as diluent.

The tests were not designed for intercomparison of the treatments at different pH levels, but a rough comparison can be made since water was included as a diluent in each experiment. Such comparison indicates that the best diluent to use is cysteine at pH 5 or 4. Apparently, pH is less important than the compound used as diluent, at least from pH 4 to pH 6.

5) *Extraction methods.*—The 3 following tests were made to determine the best method of extraction. Inocula were prepared 1) by freezing ground clover leaves for 24 hours and diluting the brei (after thawing) 1:5 with water; 2) by freezing diluted ground clover tissue; 3) by freezing whole leaves that were ground and diluted after thawing; and 4) by diluting ground tissue without freezing. These 4 inocula were applied to *G. globosa* in an incomplete block design. The numbers of lesions induced in one test by the 4 inocula applied to 24 test leaves were as follows: 1) 536, 2) 1085, 3) 988, and 4) 706. In a second test, the respective lesion numbers were 755, 1411, 1741, and 1145. Statistical analysis of the combined results indicated that the differences between means are statistically significant (1% level) in the following comparisons: 4>1; 2 or 3>1 or 4; and 3>2. These results indicate that freezing favors extraction provided the material that is frozen consists of intact leaves or diluted ground tissue. The freezing of ground tissue before dilution seems deleterious, perhaps because of excessive oxidation, as indicated by darkening of the extract.

The effect on activity of clarifying the crude 1:30 water extract of fresh clover leaf tissue for 1 hour in a Servall SP centrifuge (5000 rpm) was determined. The extracts were compared on *G. globosa*, each on 24 leaves in the 2-solution scheme for direct comparison of 2 treatments. The inocula tested were 1) water added to fresh leaves before grinding; 2) water added to fresh leaves, and the extract frozen after grinding; and 3) same as treatment 2 except that the extract was centrifuged before freezing. The thawed clarified extract induced the most lesions (5.4 lesions/leaf), and the nonfrozen nonclarified extract from fresh leaves induced no lesions. The effect of treatment 2 was intermediate (3.5 lesions/leaf). These differences, though small, were statistically significant at the 1% level. The advantage of clarifying the crude diluted extract may be due to the removal of inhibitors and or oxidants in the material sedimented by centrifugation.

6) *Darkening of test plants.*—Since shading or complete darkening before inoculation increases the sensitivity of some assay plants to certain viruses (1, 2), the effect of darkening on the sensitivity of *G. globosa* to RCVMV was determined. In each of 2 tests, 1) 3 plants of *G. globosa* were darkened 24 hours before inoculation; 2) 3 were darkened 24 hours after inoculation; 3) 3 were darkened before and after inoculation; and 4) 3 were kept under continuous illumination for 24 hours before inoculation and for 24 hours after inoculation. The last group served as a control. All groups were inoculated uniformly with a standard inoculum. The total number of lesions on leaves from the several groups in one test were as follows: 1) 39, 2) 187, 3) 29, and 4) 144. All differences were statistically significant at the 1% level. The second test gave essentially the same results. The results differ from most previously reported ones in that darkening before inoculation was detrimental, whereas darkening after inoculation was favorable.

7) *Seasonal variation in apparent infectivity.*—Assays made at different times during the year indicated a seasonal variation in the number of local lesions produced in *G. globosa*. The apparent infectivity of 1:5 aqueous extracts of fresh infected red clover leaves was highest in midwinter and approached zero in late spring. The data obtained are insufficient to indicate the reason for this seasonal fluctuation, but any of several factors may be involved. Since RCVMV is rather unstable, it is possible that the virus content of the source plants is affected by seasonal changes in greenhouse temperatures and/or light intensity. It is also possible that there are seasonal variations in the amount of inhibitors present in the source plants or that plant extracts vary with the season in respect to the extent to which they enhance the oxidation of the virus following extraction. Another possibility is that the susceptibility of the test plants may be affected appreciably by seasonal changes in temperature and/or light intensity.

Physical properties of the virus.—1) *Dilution end-point.*—Ramamurthi, Ross, and Roberts (20) reported that crude juice of red clover diluted 1:100,000 induced a few lesions in some experiments. Hagedorn and Hanson (7) reported a dilution end-point of 1:1000 in most experiments, and one of 1:10,000 in a single case. In the present work, a total loss of infectivity was recorded at 1:50; aqueous dilutions of 1:30, 1:10, and 1:5 respectively gave 2, 4, and 9 lesions. Since these tests were made in late spring, the very low infectivity might have resulted from the seasonal factors discussed in an earlier section. It appears, however, that this would not account entirely for the differences between the titers found here and those reported by Ramamurthi, Ross, and Roberts (20), for some of their tests were made during late spring and summer (personal communication, D. A. Roberts). Possibly, a strain incapable of reaching high titers during summer months was inadvertently selected during biological purification of the strain used here. It is also possible that the differences are due to different clones used as source plants.

2) *Longevity in vitro.*—The longevity *in vitro* of a 1:5 aqueous extract of fresh leaves was in agreement with published reports (14, 24); at 25°C, activity was lost in 2 days in most tests but was occasionally retained for 3 days.

Aging at 25° was also tested in the presence of phosphate buffer, acetate buffer, and cysteine at pH levels of 4, 5, and 6 (all of 0.1*M* concentration). Also, survival of the virus in frozen extracts was determined. For these tests, 1:5 extracts were prepared from

TABLE 2.—*Relative infectivity, as indicated by bioassays on Gomphrena globosa, of red clover vein mosaic virus in 1:5 extracts prepared with different diluents and stored for different periods at 25° and −10° C*

Diluent	pH	Mean no. of lesions/leaf[a] after storage for indicated no. of days at indicated temperature						
		0 Day	1 Day		2 Days		3 Days	
			25°	−10°	25°	−10°	25°	−10°
Water	5.3	1.0	0.1	0.8	0.1	0.5	0.0	0.2
0.1*M* cysteine	4.0	13.7	0.4	0.8	0.4	1.0	0.1	0.5
0.1*M* phosphate buffer	4.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0
Water	5.3	1.5	0.3	0.7	0.1	0.5	0.0	0.2
0.1*M* cysteine	5.0	15.0	1.0	2.2	0.5	1.8	0.3	1.0
0.1*M* phosphate buffer	5.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0
Water	5.3	1.5	0.3	0.8	0.1	0.5	0.0	0.2
0.1*M* cysteine	6.0	8.8	0.5	1.5	0.5	1.1	0.1	0.5
0.1*M* phosphate buffer	6.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0

[a] At 0 days, the inoculum diluted with water was compared with each of the others on opposite leaves (each on 48 leaves). In the other comparisons, the inoculum stored at 25° was compared with the corresponding one stored at −10° on opposite leaves (each on 32 leaves). At each pH level, the differences in longevity due to diluents and those due to temperature were statistically significant at the 1% level (*F*-test). Inocula prepared with acetate buffer (pH 4, 5, and 6) induced no lesions.

fresh red clover leaves in 3 different diluents: phosphate buffer, acetate buffer, and cysteine (all of 0.1*M* concentration and at the same pH level in a single test). Extracts were also prepared with water, which served as controls in each test. Immediately after extraction, each extract was compared with the control in infectivity tests on *G. globosa*. Each of the different extracts was then divided, and half was stored in a 25° constant-temperature chamber and the other half in a freezer at −10°. On the first, second, and third days after extraction, assays were made in which the frozen and nonfrozen portions of each extract were compared on opposite leaves of *G. globosa*. The replicates of each experimental set gave results consistent with those of the original experiment.

None of the preparations prepared in acetate buffer were active. When water or cysteine was used as diluent, frozen samples retained activity better than did those stored at 25°, regardless of the time interval (Table 2). The inocula prepared with phosphate buffer showed no activity after the first day at either temperature. At each pH level, cysteine was superior to water as a diluent. With all preparations, loss in activity increased as the storage period increased.

Even though the tests were not designed for intercomparing the different pH levels, such comparisons seem valid since the interactions between plants, between days, and between replicates were not statistically significant. Thus, the diluent that best preserves virus activity appears to be cysteine at pH 5. This was true for both frozen and nonfrozen extracts, with the latter being inferior to the former. Cysteine was least effective at pH 4, with cysteine at pH 6 intermediate. At all pH levels cysteine preserved activity better than did water, both at 25° and at −10°. Water was superior to phosphate or acetate buffers at any pH level. Again, the results indicate that the compound used is of greater importance than pH in preserving virus activity. Inocula diluted with cysteine always remained bright green, whereas inocula diluted with water, with acetate buffer, or with phosphate buffer turned dark after extraction.

3) *Inclusion bodies.*—In a search for inclusion bodies in leaf hairs of red clover, the staining method of McWhorter (15) was followed; the stains used were 0.5% phloxine and 0.5% trypan blue. Equal numbers of mounts from healthy and from diseased leaves were examined, the leaves ranging from some that were just unfolding to some that were about 5 weeks of age. Inclusion bodies were never found in healthy leaves but were easily detected in diseased leaves of all ages. They were either amorphous or crystalline, the amorphous type being the more prevalent. The crystalline bodies were either rectangularly 4-cornered, with all sides of equal length, or more or less hexagonal. The dimensions of the 4-cornered bodies averaged 6.4×6.4 μ, and those of the hexagonal ones 6.4×8.0 μ. Most of the amorphous inclusion bodies had a diameter of 12.8 μ or less.

Physiological studies.—1) *Longevity of infected plants.*—The effect of RCVMV infection on the vigor of red clover plants, especially on longevity in the greenhouse, was determined in an 8-month observation period extending from August, 1957, through March, 1958. Cuttings were taken from 2 healthy and from 3 diseased red clover plants, giving 5 clonal groups. The diseased cuttings did not root as well as did healthy ones (Table 3); of the diseased cuttings

TABLE 3.—*Rooting of cuttings, and survival in the greenhouse of established plants, from 2 healthy and 3 diseased (red clover vein mosaic virus) clonal groups of red clover*

Status and clone no.	No. of cuttings taken	No. of plants established	Percentage survival at 8 months
Diseased, 1	30	25	32
Diseased, 2	30	25	17
Diseased, 3	24	19	27
Healthy, 4	25	25	100
Healthy, 5	25	25	88

TABLE 4.—*Comparative fresh weights, rates of apparent photosynthesis, and respiratory rates of healthy and infected (red clover vein mosaic virus) red clover tissue*

Weeks after inoculation	Clone No.	Clone Condition	Symptoms[a]	Fresh weight (g/g DW)[b]	Gas uptake (μl/minute/g DW)[b] CO_2	Gas uptake (μl/minute/g DW)[b] O_2
3	1	Healthy		5.75	19.33	5.37
		Diseased	VS	9.38	15.38	10.93
	2	Healthy		5.63	19.57	6.78
		Diseased	VS	8.69	15.99	11.44
6	1	Healthy		4.66	12.11	4.00
		Diseased	S-P	5.25	8.88	4.35
	2	Healthy		4.33	16.30	5.43
		Diseased	P	5.19	9.35	5.98
9	1	Healthy		5.50	13.79	4.69
		Diseased	P	6.02	9.58	5.09
	2	Healthy		5.41	17.50	4.82
		Diseased	P	5.82	11.35	5.06

[a] VS = very slight, S = slight, P = pronounced.
[b] DW indicates dry weight. Fresh weight data based on 1 replication, gas exchange data on 4.

that did root, fewer than 30% survived for 8 months, whereas almost all of the healthy plants survived. Presumably, the virus would also affect longevity under the more adverse conditions in the field. Reports in the literature (16, 19, 22) indicate that this is the case. The differences between the clones suggest that it might be possible to find clover varieties that are tolerant to the virus.

Since RCVMV was found to affect longevity of red clover under rather favorable greenhouse conditions, the work was extended to studies on the effect of very high temperatures on diseased plants. Four plants of one clone (2 healthy and 2 that had been inoculated about 6 weeks previously and were showing severe symptoms) and 2 additional old plants (about 2 years old, one healthy and one diseased) were exposed for 72 hours at a temperature of 38°C and at 100% relative humidity in an illuminated controlled-temperature chamber. They were then placed back on the greenhouse bench for observation. The diseased plants, both old and young, died soon after the treatment, whereas the healthy ones showed no injury. The experiment was repeated with different clones at a later date, and only one healthy plant survived. Since the 2 tests were made at different times of the year, it seems that ability to withstand high temperature is affected not only by virus infection and clonal differences but also by the predisposition of the plants.

2) *Effect of RCVMV infection on physiological processes.*—The study of the effect of RCVMV on the physiological processes of red clover plants 3, 6, and 9 weeks after inoculation involved determinations of the rate of apparent photosynthesis, rate of respiration, and fresh and dry weights of healthy and diseased plant samples.

The experimental material consisted of 2 clonal groups, with 8 plants each. The clones were obtained from 2 different healthy red clover plants. Each group of 8 plants was divided into 2 groups of 4 plants. Four plants of each of the clonal groups were used as healthy controls, whereas the other 4 plants were inoculated with a 1:30 aqueous virus-containing extract on February 12, 1958, 3 weeks before the first determination.

The rate of photosynthesis and of respiration was measured manometrically by the Warburg method described by Umbreit, Burris, and Stauffer (26). Two leaf disks of 1-cm diameter were punched from one trifoliate leaf of each of the 4 diseased and each of the 4 healthy plants within each clonal group. One of each pair was placed in a Warburg flask for measurement of apparent photosynthesis, and the corresponding disk was placed in a flask for determination of the rate of respiration, the lower epidermis of the disk facing upward in both cases. For both measurements, the center well and the sidearm of the vessel contained 0.1 ml 0.25*M* bicarbonate buffer to provide a constant carbon dioxide pressure.

Attachment of the Warburg flasks to the manometers was followed by a 15-minute period of equilibration in a constant-temperature water bath of 30°C. Actual change in pressure (mm) was then taken for the 2 flasks every 15 minutes and recorded as the rate of respiration or apparent photosynthesis, respectively, per minute per dry-weight unit.

Photosynthesis proceeded in an illumination of 1000 ft-c provided by fluorescent lights underneath the glass bottom of the water bath. Respiration took place in complete darkness.

Weight determination involved the harvesting and weighing of the whole foliage of the experimental plants, which was then dried at 100°C in a constant-temperature oven for 48 hours and then weighed.

The data on healthy tissue (Table 4) indicate seasonal variations in rates of respiration and apparent photosynthesis. Variations in the data for the 2 clones are probably attributable to the heterogeneity of the clover variety.

Virus infection had a pronounced inhibitory effect on apparent photosynthesis. This effect was least noticeable 3 weeks after inoculation, probably because symptoms had not yet developed fully. Since certain areas of the infected leaves become chlorotic, it is not surprising that the rate of apparent photosynthesis is lower in such leaves than in normal leaves. It is not known whether the chlorotic areas resulting from infection by RCVMV are due to destruction of chloroplasts, to inhibition of chloroplast formation, or to inhibition of their development and functioning. The fact that symptoms become masked under certain conditions and reappear later, indicates that the effect of virus infection is reversible; this suggests that the chloroplasts are not destroyed. It is possible, however, that virus infection destroys chloroplasts, but does not permanently affect the ability of cells to produce them.

Infection by RCVMV increased the rate of respiration (Table 4); this effect was most pronounced 3 weeks after inoculation. This coincides with the onset of symptom development and probably coincides with

the period in which the rate of virus synthesis is highest.

From a practical point of view, the experiment indicated that the virus would have an adverse effect on hay yield. It also points to the possibility of finding tolerant clover clones, since there were significant differences among the clones used.

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EFFECT OF PLANT RESIDUES ON ROOT ROT OF BEAN[1]

William C. Snyder, Milton N. Schroth, and Theodosios Christou[2]

SUMMARY

Fusarium solani f. *phaseoli* and, to some extent, *Rhizoctonia solani* and *Thielaviopsis basicola*, the principal causal agents of bean root rot in the Salinas Valley, were controlled in greenhouse tests by adding to field soil organic amendments of a high carbon-to-nitrogen (C:N) ratio. Control was achieved with mature barley straw, wheat straw, corn stover, and pine shavings. Mature barley straw gave good control, but green barley hay (with higher nitrogen) increased the disease severity. The benefits of mature barley straw were negated by adding nitrogen. Soybean and alfalfa residues that have a low C:N ratio increased root rot.

The bean root rot problem in the Salinas Valley (California) has grown steadily worse with the intensive growing of small white dry beans, *Phaseolus vulgaris* L. The principal causal agents are *Fusarium solani* f. *phaseoli* (Burk.) Snyd. & Hans., *Rhizoctonia solani* Kühn, and, early in the season, *Thielaviopsis basicola* (Berk.) Ferraris. The pathogens, in varying proportions, infect the roots and hypocotyls, causing a cortical rot.

When first used for beans, this land often produced as high as 30–40 100-lb. sacks per acre. With repeated culture of beans, yields declined to the present average, 20 sacks or less per acre, and the root rot complex has increased in seriousness. Higher yields, around 40 sacks per acre, have frequently been on acreage previously planted to barley. Growers also noticed that, after bean production had declined because of the disease complex, rotation with barley appeared to reduce disease severity and increase yields. Consequently, a study was initiated of the disease complex and its control by using barley straw as a soil amendment.

MATERIALS AND METHODS.—The soil used was a sandy loam obtained from a bean field in the Salinas Valley. The beans, grown in 2-lb. perforated coffee cans in saucers, were watered from below. Mature barley straw in segments 1–3 cm long was incorporated into the soil at the rate of 1.0% by weight of dry soil. The following amendments, in an air-dried condition, were used: mature barley straw, green barley hay, mature barley straw plus 0.3% ammonium nitrate by weight, and mature barley straw plus 1% sulfur by weight. Controls contained no amendments. The soil with amendments was incubated 10 days in the greenhouse before planting. Eight beans were planted 2 in. deep in each can. Three replicates were made of each series. The plants were harvested after 4 weeks, and the severity of lesions caused by *F. solani* f. *phaseoli* and other root-rotting pathogens was recorded. Amendments of wheat straw, corn stover, pine shavings, alfalfa, and soybean residues were similarly tested.

EXPERIMENTAL RESULTS.—The hypocotyls and roots of the control plants in a representative experiment were heavily infected and densely covered with reddish-brown lesions that, under the environment imposed, were caused principally by *F. solani* f. *phaseoli*. Under other conditions in other experiments, the major pathogen was *R. solani* or *T. basicola*, or both. Hypocotyls were more severely infected than roots. Beans in soil containing mature barley straw had a few scattered lesions on the roots and hypocotyls. Ammonium nitrate with the mature barley amendment nullified the beneficial effects otherwise obtained. Roots and hypocotyls were infected about as heavily as the control. Combining sulfur with mature barley straw also reduced control, though incidence of lesions on roots and hypocotyls was about half that of the controls. Green barley hay not only provided no control but appeared to increase root rot slightly (Fig. 1).

Amendments of wheat straw, corn stover, or pine shavings gave good control, with only a few scattered lesions on roots and hypocotyls. Alfalfa or soybean residues increased disease severity, with roots and hypocotyls being completely covered with lesions. Rot was most severe with the alfalfa amendment, with most of the hypocotyls being almost severed.

These results reflect especially the reduction of infection by *F. solani* f. *phaseoli*. Infection by *R. solani* or *T. basicola*, however, was also reduced by barley straw, wheat straw, corn stover, and pine shavings. Alfalfa and soybean residues especially increased the amount of infection by *R. solani*.

DISCUSSION.—The results suggest that root-rot reduction is related to a high C:N ratio induced in the soil by mature barley straw and other amendments of low nitrogen content.

An organic amendment, rich in carbon and low in nitrogen, such as straw, presumably stimulates the growth of many microorganisms, with immobilization of soluble nitrogen resulting. The microorganisms apparently utilize the soil nitrogen in decomposing the energy source added to the soil, and *F. solani* f. *phaseoli* under these highly competitive conditions is unable to function as a pathogen. However, if nitrogen is added with barley straw, increasing the amount of available nitrogen, the pathogen again becomes active and causes severe root rot.

The differences in control between green barley hay and mature barley straw may be attributed to the difference in their composition. Green barley hay has

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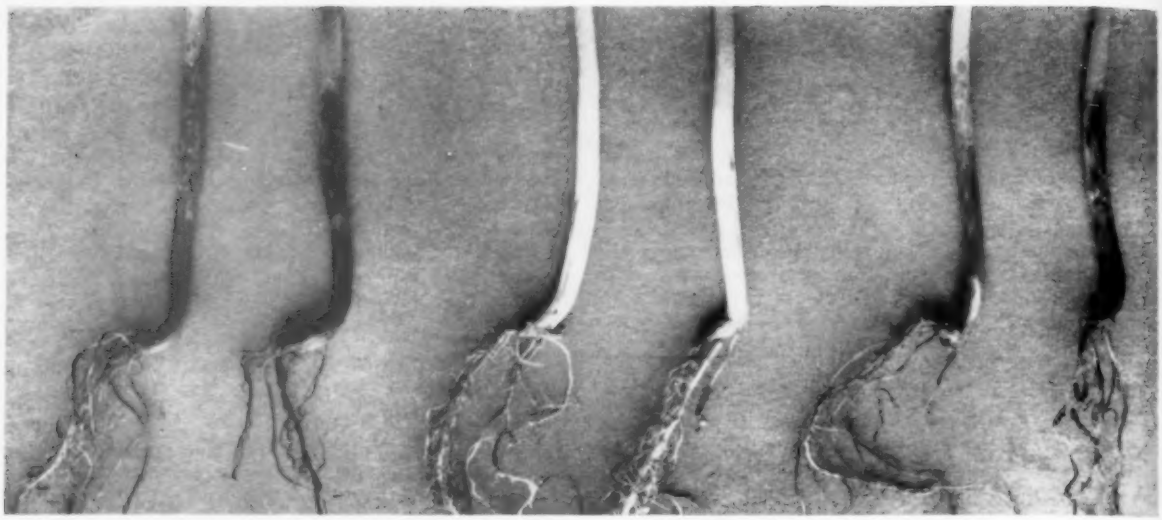

Fig. 1. Effect of adding organic amendments to field soil upon the development of bean root rot. Root rot was increased slightly **(left)** by green barley hay, and reduced **(center)** by mature barley straw. The control **(right)** had no amendment.

a smaller percentage of lignin and other constituents resistant to decomposition, a higher content of water-soluble constituents and nitrogen, and therefore a lower C:N ratio.

Wheat straw, corn stover, and pine shavings, all of which gave good control, have high C:N ratios. The C:N ratios are about 80:1 in the straws, 60:1 in corn stover, and as high as 400:1 in pine shavings.[3]

Alfalfa and soybean residues, which slightly increased the severity of root rot, have relatively low C:N ratios, about 16:1.

Amendments with high C:N ratios also appeared to lower the amount of infection by *F. solani* and by *T. basicola*.

Murray[4] indicated that bacteria multiply rapidly when straw is added to soil and play an important role in immobilizing nitrogen. That bacteria may play such a role in the tests reported here is indicated by the reduction in disease control when sulfur was added with straw to lower soil pH from about 7.5 to about 5.0, thereby reducing bacterial activity. With slower bacterial growth the pathogens presumably had a larger supply of nutritients available and thus functioned more effectively in bringing about root rot infection.

Using organic amendments of high C:N ratios to control root-rotting organisms is more adaptable to the legumes, perhaps, than to other plants, since the stimulation of nitrogen-fixing Rhizobia upon their roots enable these plants to obtain at least some of the nitrogen they need for normal growth.

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A CONTINUOUS SPORE SAMPLER [1]

S. M. Pady [2]

SUMMARY

A new continuous quantitative slit-type spore sampler was built that gives 24 hour-long deposits on a 1×3-in slide. The main feature is a sampling column with pump attached (Dayton blower, 50 cfm). The upper revolvable half contains the slit, which has a 14×2-mm aperture less than 1 mm from the silicone-coated slide. Timing mechanism consists of 2 small electric motors (1/60 and 1 rpm) that elevate the slide once each hour. The sampler is watertight, light, and compact, and has performed satisfactorily under all weather conditions. At a flow rate of 0.4 cu. ft./minute the efficiency is at least 50%. Preliminary studies of leaf and stem rust, Helminthosporium, Alternaria spores, and hyphal fragments show definite hour-to-hour variations with peaks up to 50 spores /cu. ft., occurring mostly in the afternoon, with occasional peaks at night.

Aerobiological investigations of plant pathogens, particularly of the dissemination and concentration of fungus spores, are valuable in understanding the epidemiology of plant diseases (2, 18). Although the occurrence of spore showers is generally accepted, there are few data on their concentration in the air. Adhesive-coated slides exposed for 24 hours are commonly used, particularly in the study of cereal rusts (11, 12, 13). Quantitative samplers, as the Bourdillon slit sampler (1), G. E. electrostatic sampler (7), and the Pady-Rittis slit sampler (8), provide additional data. Little is known, however, of spore density throughout a 24-hour period. Hirst (4) devised a spore trap, a single slide moving slowly past a slit orifice, that gives a continuous quantitative record of the spores in the air. Panzer, Tullis, and Van Arsdel (17) constructed a sampler with 24 slides, each slide receiving an hour's deposit. It seemed desirable to construct a slit sampler that would deposit spores in 24 one-hour bands on a 1×3-in. slide. This paper describes such a sampler, which is compact, light, and watertight, and has performed satisfactorily through the 1957 and 1958 growing seasons. A preliminary report has already appeared (10).

The complete sampler is shown in Fig. 1. Basically, it consists of a sampling column (1:4, 1:5) with pump attached directly to the column, both being mounted on a wooden frame and base (1:8). The upper rotatable part of the column (1:4) contains the slit assembly (1:3), which is protected by a rain shield (1:2) and held toward the wind by a wind vane (1:1). Top and side covers (1:6, 1:7) protect the pump and timing mechanism.

In Fig. 2 the wind vane (2:1), rain shield (2:2), slit assembly (2:4), side and top cover (2:17), and outer cover of the sampling column (2:3) have been removed to show internal parts. The slit assembly (2:4) consists of a flattened tube, 50×20×8 mm, with an internal orifice 14×2 mm. A curved flange on the slit fits snugly against the cover and is held in position by a lock bar and nut. The outer end, which faces the wind, is beveled to a knife edge. The opening for the slit assembly can be seen in the side of the column cover (2:3). The pump (Dayton Blower 110V, 60C, 50 cfm) (2:11) and motor (2:13) are held in position by 2 metal straps (2:12). Inside the column is the motor mount (2:5) and elevator motor (2:6) with slide holder and slide.

The timing mechanism, fastened to the frame, under the motor, consists of an electric motor (2:15, 1/60 rpm) and a relay that activates the elevator motor (2:6, 1 rpm). The motor (2:15) is fastened to a metal plate with a 2-in. disk mounted on the motor shaft and a short rod soldered to the edge of the disk. The relay (2:14) is positioned on the plate so the rod contacts the relay button. Adjustment screws on the relay permits lengthening or shortening the contact time to permit satisfactory spacing of bands. A manual switch (2:16) activates the elevator motor for removal and insertion of slides.

In Fig. 3 the components of the sampling column have been separated and arranged to show their relationships. A 1×3-in. microscope slide (3:5) is inserted in a removable slide holder (3:4) and held in place by strong brass spring clips. The motor mount (3:3) has grooves on the front to carry the slide holder, and the elevator motor (3:6) is attached at the rear. The holder has a rack that meshes with a pinion gear on the elevator motor. A screw (not shown in the photograph) fastens the motor mount securely to the column (1:8). When the slide holder is in position, the top of the slide is opposite the slit orifice and less than 1 mm from the slide. Wires from the elevator motor soldered to 2 contact rings (3:10) mounted on a Lucite ring (3:9) provide a continuous connection with electrical contacts (3:11) in the fixed base (3:16) and lead-in wires (3:19) from the timing mechanism. A slotted sleeve (3:12), attached to the upper column,

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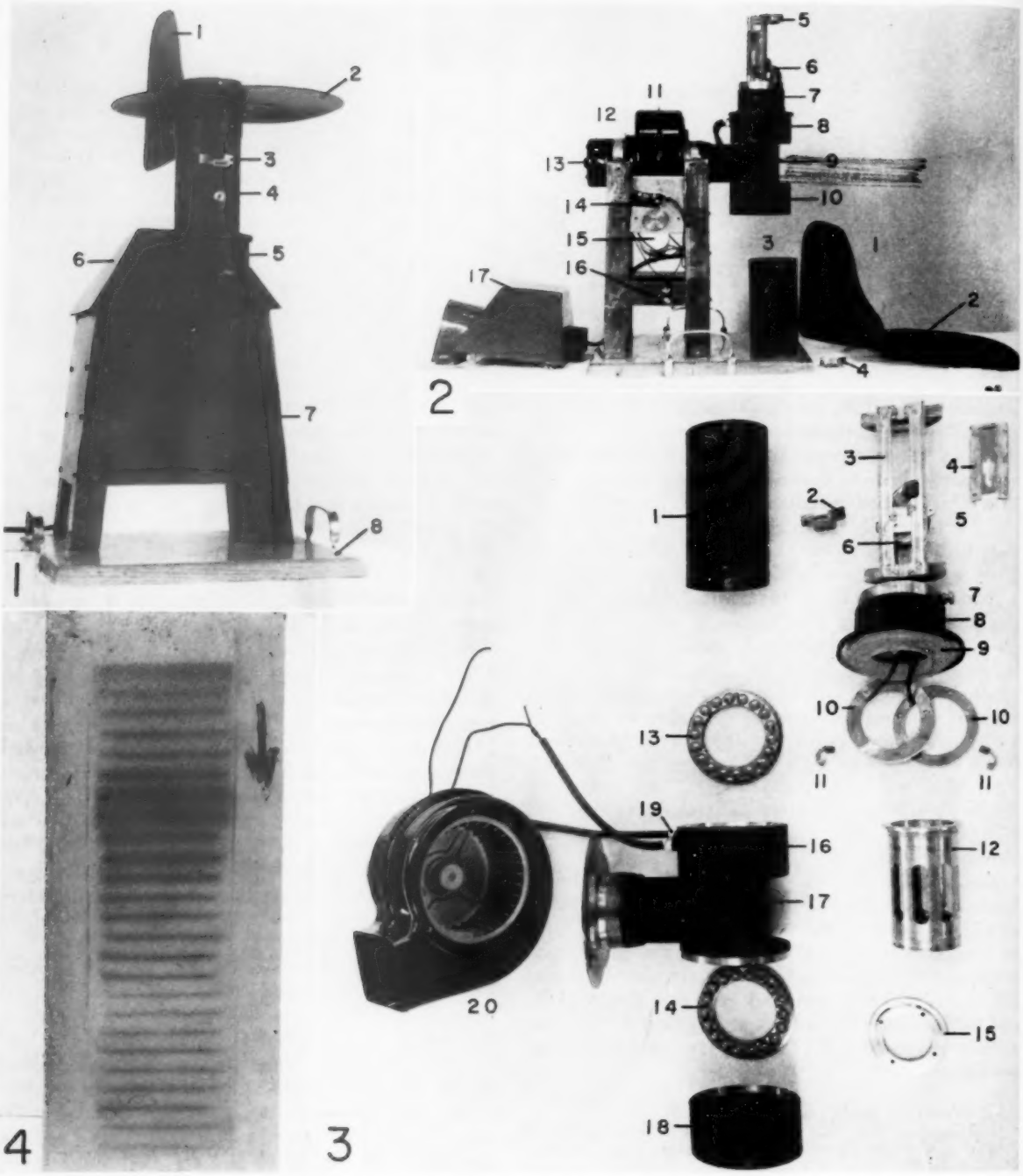

Fig. 1-4. Complete sampler. **1)** Wind vane, **2)** rain shield, **3)** slit assembly, **4, 5)** movable and fixed portions of sampling column, **6)** top cover, **7)** side cover, **8)** base. **Fig. 2.** Sampler with covers removed. **1)** Wind vane, **2)** rain shield, **3)** column cover, **4)** slit assembly, **5)** motor mount, **6)** Elevator motor, **7)** base of upper movable portion of column, **8, 9, 10)** base of sampling column, **11)** squirrel cage blower, **12)** metal holding straps, **13)** motor of Dayton blower, **14)** relay, **15)** timing motor, **16)** manual toggle switch, **17)** cover. **Fig. 3.** Exploded view of sampling column and blower. **1)** Cover, **2)** slit assembly, **3)** motor mount, **4)** slide holder, **5)** glass slide, **6)** elevator motor, **7)** nut to hold cover, **8)** base sampling column, **9)** Lucite ring, **10)** contact rings, **11)** electric contacts, **12)** slotted sleeve, **13)** upper roller bearing, **14)** lower roller bearing, **15)** bearing ring, **16, 17, 18)** parts of lower fixed portion of sampling column, **19)** lead-in wires, **20)** Dayton blower. **Fig. 4.** Photograph of slide No. 336 exposed July 4-5, 1957, showing typical bands.

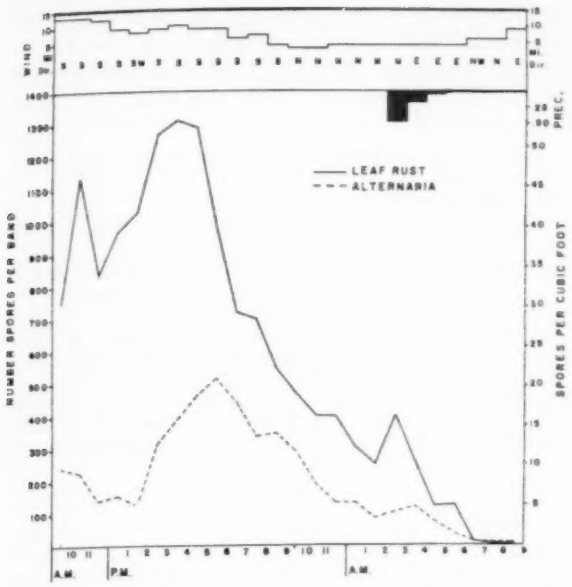

Fig. 5. Numbers of leaf rust and Alternaria spores, June 7-8, 1957.

extends into the fixed base (3:16, 17). Roller-bearing rings (3:13, 14) allow freedom of movement, and the slotted sleeve permits unrestricted air flow. The pump (3:20) is fastened to a plate soldered to the base of the column, and all joints are airtight and watertight. The flow rate is 0.4 cu. ft./min. (11.3 liters/min.).

The sampler was bolted to a platform table, 150 ft. above the ground, on the roof of a campus building, and there was free flow of air around the platform. Electric outlets under the platform provided the necessary current. Slides, changed between 8:30 and 9:00 A.M. each morning, were coated with silicone grease (DC-4). A typical slide is shown in Fig. 4.

Slides may be read under low or high power. Large spores such as the rusts, Alternaria, and Helminthosporium are readily observed under low power without a cover glass. In practice it was found best under low power to examine an entire band rather than just a few fields. Numbers per cubic foot were determined by dividing the number of spores per band by the air volume. One disadvantage encountered is that dust concentrations in the air may cause such heavy dust deposits that spores may be obscured (Fig. 4).

The size of the slit and the distance from the slide are the same as in the Hirst (4) spore trap, which has a minimum efficiency of 45% at 17.5 liters/minute and 70% at 10.0 liters/minute. The efficiency of this sampler at 0.4 cu. ft./min. (11.3 liters/min.) is at least 50%.

RESULTS.—In Fig. 5 and 6 spores of leaf rust (*Puccinia rubigo-vera* (DC) Wint. f. sp. *tritici* (Eriks.) Carleton), Alternaria, Helminthosporium, stem rust (*P. graminis* Pers. f. sp. *tritici* Eriks. & E. Henn.), hyphal fragments, and meteorological data on wind and precipitation are given for June 7 and 8, 1957. In all cases there was a distinct hour-to-hour variation, but with a maximum in the afternoon and with one or more minor peaks. With a continuous south wind, leaf rust urediospores reached a peak of 55/cu. ft. at 3 P.M., followed by a sharp decline (Fig. 5). A wind change to the north occurred at 10 P.M., followed by heavy rain at 3 A.M., with light rain continuing to 9 A.M. The effectiveness of precipitation in removing spores from the air is well illustrated in the two figures. There is evidence also that the air mass that moved in at 10 P.M. had a much lower concentration of leaf rust spores (Fig. 5) and stem rust spores (Fig. 6).

The slides read are insufficient to determine whether or not these results are typical. Leaf rust urediospores have been examined in 17 slides; of these, 11 had a single peak, 8 an afternoon peak at 3–5 P.M., 3 an early morning peak at 1–5 A.M., whereas 6 had two peaks, one in the daytime, the other at night. Of 9 stem rust slides, 5 had single daytime peaks between 1 and 5 P.M., and one at 7 A.M., whereas 3 had two peaks, one in the daytime, the other at night. Results of these studies in 1957 and 1958 will be reported later.

DISCUSSION.—Continuous quantitative samplers are a relatively new development in the field of aerobiology. The Hirst spore trap (4) and the sampler of Panzer, Tullis, and Van Arsdel (17) are making possible basic studies in the biology of the airborne fungi and are providing information on the dissemination of fungus spores. The results obtained by both of these samplers clearly indicate a great variation in numbers of spores throughout a 24-hour period. In England, Hirst (5) found that most spores showed a well marked diurnal periodicity, with Alternaria, Cladosporium, Erysiphe, smuts, and rusts having a peak in the afternoon.. At night, and following rains,

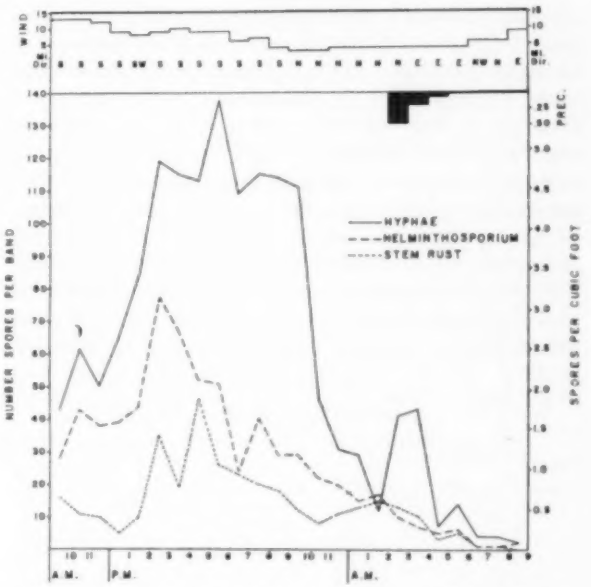

Fig. 6. Numbers of hyphal fragments, Helminthosporium and stem rust spores, June 7-8, 1957.

hyaline spores, especially basidiospores and ascospores, were most numerous. Panzer, Tullis, and Van Arsdel (17) found a similar pattern, with Alternaria, Penicillium, Nigrospora, Tricoconis, and Curvularia having daytime peaks, and Cladosporium, mildew, Leptosphaeria, Epicoccum, Piricularia, and Diplodia having a maximum at night. Gregory and Sreeramulu (3), in England, and Waggoner and Taylor (19), in the United States, obtained similar results with the Hirst spore trap.

Spore numbers in Kansas have been found to be seasonal, with highest numbers in July and August and lowest numbers during the winter (9). The highest total spore concentration recorded to date was 2866/cu. ft. 6/20/58. Total units (2 or more spores in a group) were 1771/cu. ft., with Cladosporium 81 per cent of the total. These data were obtained from a single 30-min. exposure between 8:30–9:30 A.M. If diurnal periodicity occurs in Kansas, it follows that this morning figure is low and would have been higher had the sample been taken in the afternoon. The use of continuous samplers permits the determination of spore loads throughout the day, and the exact time and duration of maximum spore load. Hirst (5), using his spore trap, found concentrations of Cladosporium as high as 60,000 units/m^3 and hyaline basidiospores 270,000/m^3. Gregory (3), using a Hirst spore trap, obtained concentrations of Cladosporium to 100,000/m^3 and Sporobolomyces as high as a million/m^3 following a rain. Waggoner and Taylor (19) used the Hirst spore trap in Connecticut to study dissemination of *Peronospora tabacina* Adam spores from infected tobacco beds. They obtained maximum spore loads of 432–5000/m^3 (12-145/cu. ft.) between 8:30–9:30 A.M. with the sampler 46 cm above the beds. The leaf rust spore concentration of 55/cu. ft. (Fig. 5) was obtained with the sampler 150 ft. from the ground, at least 1 mile from the nearest wheat field and 75–100 miles from the nearest area of concentrated wheat production. More information is needed on spore loads carried by the air at various distances from the source of infection. The continuous-type samplers should provide the data.

Hyphal fragments not only have been found to be present in the air throughout the year but also appear to display diurnal periodicity (Fig. 6). They have been found by the author in the Arctic (14), Canada (16), over the Atlantic ocean (15), and in Kansas (9). In England, only one investigator (6) has mentioned their occurrence. Their importance is indicated by their presence in numbers up to 52.8 cu. ft. (9) and also by the recent discovery that these hyphae are viable and hence capable of producing a colony or causing infection. It is believed that these hyphal fragments represent an important means of fungal propagation that has been largely overlooked.

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A DISEASE OF CARNATIONS CAUSED BY THE NEMATODE CRICONEMOIDES XENOPLAX[1]

S. A. Sher

SUMMARY

Criconemoides xenoplax Raski can cause a disease of carnations characterized by a reduced root system, stunted top growth, and reduction in number of blooms. The disease was eliminated by growing plants in fumigated soil. Infestation of soil with *C. xenoplax* produced the disease. A *Paratylenchus* sp. from the same soil had no significant effect on carnation plants. The name, ring nematode decline of carnations, is proposed for this disease.

Nematodes were first reported as a possible cause of disease in carnation (*Dianthus* sp.) in 1881, by Berkeley (1). In 1894 root-knot nematode (*Meloidogyne* sp.) was first recorded as a disease of carnations (11). Chitwood recorded *Meloidogyne javanica* attacking carnation in 1949 (3), and Sasser in 1954 demonstrated that 3 other species of rootknot—*M. incognita*, *M. hapla*, and *M. arenaria*—can parasitize carnation, *M. hapla* causing only an extremely light infection (9). Other species of plant-parasitic nematodes reported to parasitize carnations are *Heterodera schachtii* (6), *Heterodera trifolii* (8), and *Pratylenchus pratensis* (4). Recently Jenkins and Taylor described *Paratylenchus dianthus* as a possible factor in the poor growth of carnations (5).

Soil samples from carnation nurseries in southern California often contain *Meloidogyne incognita*, a *Paratylenchus* sp., and *Criconemoides xenoplax* Raski. The rootknot nematode has been the only nematode pest of carnations recognized by southern California growers. Because species of the genus *Paratylenchus* are known plant pests (7), and *Criconemoides xenoplax* feeds on plant roots (10) and is reported as a possible plant pest (2), a preplant fumigation test was applied to soil from a nursery containing *Paratylenchus* sp. and *C. xenoplax*. The results obtained from this test led to further investigation to ascertain whether *C. xenoplax* could limit the growth of carnations.

SOIL FUMIGATION EXPERIMENT.—*Materials and methods.*—Soil (sandy loam) from a carnation nursery in Los Angeles containing a *Paratylenchus* sp. and *Criconemoides xenoplax* was used to fill six 3-gal. glazed crocks. Two crocks were treated with D-D (1,3-dichloropropene; 1,2-dichloropropane), 3 ml per crock; 2 crocks were treated with chloropicrin (trichloronitromethane), 3 ml per crock; and 2 crocks were untreated. The fumigants were pipetted into the center of the crock, and the soil tamped down lightly. A ½-in. water seal was used on the chloropicrin treatments. Three weeks after treatment the soil was transferred to 36 4-in. clay pots, making 12 pots in each series. One-month-old rooted carnations (*Dianthus caryophyllus* var. Alenius) were planted in each pot, and the pots were randomized on a greenhouse bench.

Two months later, the plants were cut back to 6 in., and remaining side branches were pruned and their dry weights taken. Observations were made of the tops and roots of the plants 6 months after the start of the test. Two 50-cc soil samples were obtained from each pot and put on a modified Baermann funnel for 3 days, and the nematodes obtained were counted. Roots from each series were cultured for fungi. Dry weights were taken of the entire plant.

RESULTS.—Plants grown in both fumigated series were noticeably larger at the termination of the test than were plants grown in untreated soil (Table 1). There were no observed differences in the plants grown in the chloropicrin-treated soil and the D-D treated soil. A *Paratylenchus* sp. was recovered from 11 of the 12 replications in the D-D treated plants. A *Paratylenchus* sp. and *C. xenoplax* were recovered in all the replications of the untreated soil. Nematodes were not recovered in the chloropicrin series (Table 1). A *Fusarium* sp. and a *Rhizoctonia* sp. were recovered from the roots of all 3 series.

SOIL INFESTATION EXPERIMENT.—*Materials and methods.*—One-month-old rooted carnation cuttings (var. Pink Sim) were planted into 40 4-in. clay pots containing steam-sterilized sandy loam. Ten pots were infested with about 6000 *Paratylenchus* sp. per pot; 10 pots with about 6000 *C. xenoplax* per pot; 10 pots with washings that had contained *Criconemoides* and *Paratylenchus*; and 10 pots with 50 ml of distilled water. The washings series was obtained by pouring the water containing the nematodes (a *Paratylenchus* sp. and *Criconemoides xenoplax*) through a 325-mesh screen 5 times to remove the nematodes and collecting the water in a pan. Fifty ml of this was poured around

TABLE 1.—*Effect of preplant soil fumigation on control of nematodes and growth of carnations*

Treatment	Dry weight[a] (g)	Nematodes per 100 cc of soil	
		Paratylenchus	Criconemoides
Chloropicrin	20.8**	0	0
D-D	19.4**	192.7	0
Untreated	10.6	66.3	151.6

[a] ** = Significant at .01 level over the untreated plants.

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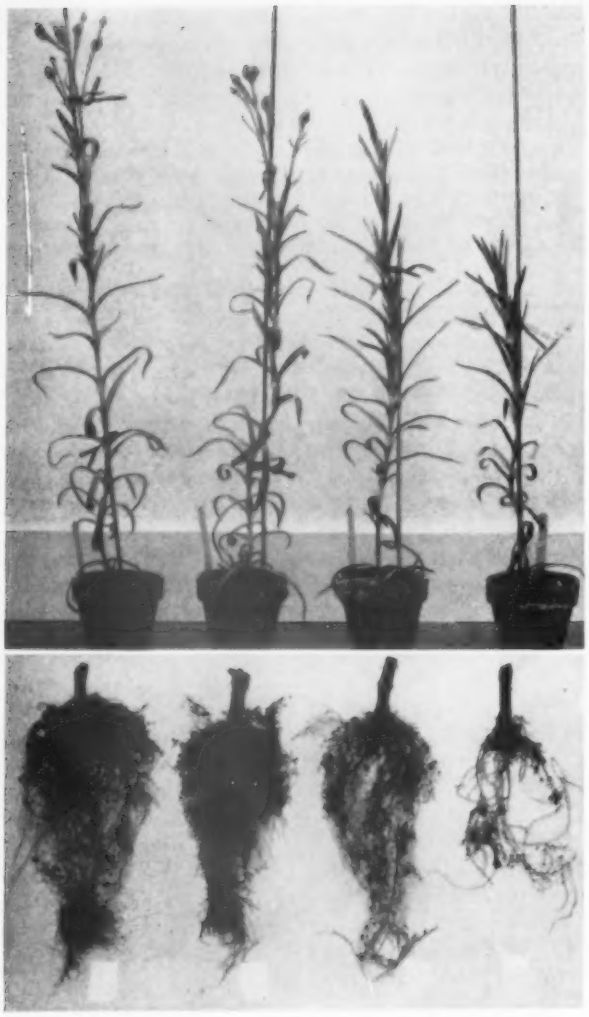

Fig. 1. Tops and roots of carnation plants grown in steam-sterilized soil. From left to right, no treatment, washings from which nematodes have been removed, a *Paratylenchus* sp. added, *Criconemoides xenoplax* added.

each plant as a possible check on associated microorganisms. The pots were randomized on a greenhouse bench.

The nematodes used in this test were obtained by infesting 6 pots with 100 *C. xenoplax* each, and pots with 100 *Paratylenchus* sp. each from the previous test. The nematodes were individually picked out, put into distilled water, and poured around the roots of carnations growing in steam-sterilized 5-in. clay pots. Three months after infestation of the pots the soil was washed through soil screens and put on modified Baermann funnels for 3 days. An average of 12,000 *C. xenoplax* were recovered per pot, and 41,000 *Paratylenchus* sp. per pot.

Two months after infestation with nematodes, all the plants were cut back to 6 in., and all side branches were removed, and their dry weights taken individually.

The number of flowers produced by each plant was recorded. Six months after the start of the test, observations were made on the tops and roots. Roots from each series were cultured for fungi. Dry weights of the plant were taken. Nematode counts were made as in the previous test.

Results.—All the plants grown in the *Criconemoides*-infested soil were noticeably reduced in size (Fig. 1). Plants in *Paratylenchus*-infested series were smaller than in the 2 series without nematodes, but noticeably larger than those in the *Criconemoides* series. Flowers were produced by all of the plants in the untreated and washings series, only 5 of the 10 replications of the *Criconemoides* series, and 9 of the 10 plants in the *Paratylenchus series* (Table 2). Roots of the plants in the *Criconemoides* series were all markedly reduced, whereas those of the *Paratylenchus* series appeared slightly smaller than the 2 series without nematodes, but noticeably larger than the *Criconemoides* series (Fig. 1-B). High populations of a *Paratylenchus* sp. and *C. xenoplax* were recovered from all pots inoculated with these nematodes (Table 2).

Examination of the carnation roots of the *Paratylenchus*-infested series under a dissecting microscope showed numerous *Paratylenchus* sp. with their anterior ends embedded in the roots. Similarly, the *Criconemoides* series showed numerous *Criconemoides* attached to the roots.

Possible pathogenic fungi were isolated from the roots as follows: *Pythium* spp. from the series receiving no treatment and the series infested with a *Paratylenchus* sp.; a *Fusarium* sp. from the series receiving the washings from which the nematodes had been removed; and saprophytic fungi from the series infested with *Criconemoides xenoplax*. (Fungi isolations were by Lily H. Davis, Department of Plant Pathology, University of California, Los Angeles.)

Discussion.—Results of the first test indicated that *Criconemoides xenoplax* may be the cause of a disease of carnations characterized by a markedly reduced root system and stunted plant. The elimination of *Criconemoides*, but not *Paratylenchus*, by the fumigant D-D indicates that this chemical is not as effective against *Paratylenchus*.

In the second test, only *Criconemoides*-infested plants showed a highly significant reduction in growth, root system, and flower production. Although *Para-*

Table 2.—*Effect of Criconemoides xenoplax and a Paratylenchus sp. on growth and flower production of carnations*

Treatment	Dry weight[a] (g)	No. flowers	Nematodes per 100 cc soil
Criconemoides xenoplax	11.8**	2.1**	86.4
Paratylenchus sp.	18.6	4.1	321.9
Washings from nematode infested soil	20.8	7.7	0
Untreated	21.4	6.7	0

[a] ** = Significant at .01 level over other series.

tylenchus reproduced in large numbers on the roots of carnations, they did not cause a significant reduction in growth under the conditions of this experiment. Both tests indicate that this *Paratylenchus* sp. was not an important factor in growing carnations in the greenhouse, and that *Criconemoides xenoplax* can cause a disease of these plants characterized by a reduced root and top growth. The name "ring nematode decline of carnations" is proposed for this disease.

The pattern of the occurrence of the fungi isolated from the root samples of the 2 tests indicates that they were not a factor in the results obtained.

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A COMPARATIVE STUDY OF THE FUNGI CAUSING THE SPRING BLACKSTEM DISEASE OF ALFALFA AND RED CLOVER[1]

Albert H. Ellingboe[2, 3]

SUMMARY

The organisms that cause the spring blackstem disease of red clover and alfalfa were compared on the bases of the general appearance of cultures, pycnidial abundance, pycnidial formation and shape, spore size, percentage of septate spores, and the effect of temperature and nutrition on these characteristics. Studies were also made on the growth rates of the cultures both on a semi-solid medium from mycelial inoculum and single spores for inoculum, and the pathogenicity of 50 monoconidial isolates on alfalfa and red clover. By none of the above criteria could the organism on alfalfa be accurately distinguished from the organism on red clover. It is concluded that *Phoma herbarum* West. var. *medicaginis* Fckl. is the valid name for the organism, and that the name *Phoma trifolii* E. M John. & Valleau should be listed in synonomy.

Various workers during the last 100 years have described a fungus or fungi, from alfalfa, that appear to fall within the limits of the blackstem fungus. The names applied by different workers have been: *Phoma herbarum* West. var. *medicaginis* Fckl. (14, 17), *Phoma medicaginis* Malb. & Roum. (10, 13), *A. pisi* Lib. var. *medicaginis* Sacc. (15), *A. imperfecta* Peck (7), and *Phyllosticta medicaginis* (Fckl.) *Sacc.* (1). Most of these workers based their conclusions on one or a few collections of diseased material, or on one or a few isolates of the fungus. An exception was Toovey, Waterston, and Brooks (19), who studied several isolates from alfalfa, designated as *Phoma medicaginis* and *Ascochyta inperfecta*, and concluded that all of them should be included under the name *A. imperfecta* Peck.

The blackstem fungus from red clover was described as a new species, *Phoma trifolii* E. M. John & Valleau (5), in 1933. This name has been accepted by some workers; others, however, said that the host range of *Ascochyta imperfecta* includes red clover and appears to be similar in other characteristics to the pathogen of alfalfa (8, 18).

Whether the fungus that causes spring blackstem of alfalfa is the same as, or distinct from, the fungus that causes blackstem of red clover has not been clear. This fungus, or these fungi, have been described under a number of different scientific names, in some cases upon rather tenuous evidence and in other cases from material collected over a very limited time and area. It therefore appeared that one way to resolve some of the nebulosity now surrounding the taxonomic and nomenclatural problem would be to make a collection of the blackstem fungi from red clover, alfalfa, and other leguminous hosts, from a large number of locations over a wide geographic area and over a three-year span; and to study the morphology of fruiting structures and spores produced on the hosts and on culture media, the range in pathogenicity of the isolates, and the range in cultural characters and variability. This should furnish evidence for accurate judgments as to whether the spring blackstem fungi of alfalfa and red clover comprise one or more species and/or genera.

MATERIALS AND METHODS.—Over the 3-year period 1954–56, 90 mass isolates were obtained from diseased red clover from 15 locations in Minnesota and one or more locations in Illinois, Wisconsin, South Dakota, Michigan, and Iowa. During the same period, 539 mass isolates were obtained from diseased alfalfa plants from 36 locations in Minnesota and one or more locations in North Dakota, South Dakota, Nebraska, Montana, Wyoming, Wisconsin, Illinois, Ohio, Alaska, and Lethbridge, Alberta, Canada. One mass isolate was obtained from each of the following hosts in Minnesota: vetch (*Vicia villosa* Roth.), trefoil (*Lotus corniculatus* L.), sweet clover (*Melilotus officinalis* (L.) Lam.), and alsike clover (*Trifolium hybridum* L.). Eighty-nine collections of diseased red clover from 6 states (Illinois, Missouri, Wisconsin, South Dakota, Indiana, and Minnesota) and 96 collections of diseased alfalfa from 8 states (Minnesota, Iowa, Wisconsin, North Dakota, Kansas, Illinois, Indiana, and South Dakota) were made for a study of the organism as it occurs on the host in commercial fields.

Monoconidial isolates were obtained either with the aid of a micromanipulator or by the dilution method.

Cultures from the various hosts were designated as follows: alfalfa = A, red clover = RC, vetch = V, trefoil = T, sweet clover = SC, and alsike clover = AC.

The pathogenicity of isolates was tested either by inoculating individual leaves of alfalfa and red clover

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[3] The writer wishes to acknowledge his indebtedness to Dr. M. F. Kernkamp for suggesting this problem and assisting in the study. Acknowledgements are also due the NC-37 Technical Committee for assistance in collecting much of the material used.

TABLE 1.—*The number of isolates of blackstem pathogens from different hosts in each of the 7 groups based on general cultural appearance*

Group	Alfalfa	Red clover	Trefoil	Vetch	Alsike	Sweet clover
I	0	9	0	5	5	1
II	20	7	0	0	2	1
III	1	5	3	0	1	1
IV	14	2	0	0	0	0
V	7	10	0	0	0	2
VI	2	5	0	0	0	0
VII	16	15	0	0	0	2
Total	60	53	3	5	8	7

with a drop of a spore suspension using an eye dropper or by dipping the plants in a mixture of spores and mycelial fragments obtained by macerating a culture in a Waring Blendor.

RESULTS.—*Cultural appearance.*—One hundred and thirty-seven monoconidial isolates from alfalfa, red clover, sweet clover, alsike clover, trefoil, and vetch were grown at one time in triplicate cultures to determine whether the cultural characteristics of the fungus could be correlated with the host from which they were isolated. An attempt was made to classify the cultures into 7 groups on the basis of their appearance. There was no correlation of cultural types and host from which they were isolated (Table 1). In addition to the differences between single-spore isolates, some cultures were extremely unstable and sectored profusely.

One hundred and seventeen single conidia were isolated from a red clover isolate, and 323 single conidia from 3 isolates from alfalfa. Of the 117 subisolates from red clover, very few were identical, and subisolates were found that fit the description of all but one of the previously described cultural groups. An equally wide range of cultural types was present in the subisolates from alfalfa.

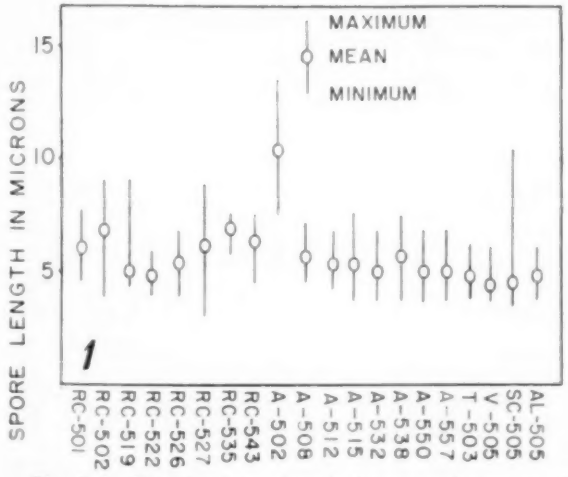
TABLE 2.—*Diameter of pycnidia of blackstem pathogen produced in 21-day-old cultures on potato-dextrose agar*

Culture no.	Host	Pycnidial diameter in microns[a] Range	Average
RC-501	Red clover	161-350	225
RC-502	Red clover	146-307	210
RC-519	Red clover	175-438	306
RC-522	Red clover	175-365	276
RC-526	Red clover	175-321	220
RC-527	Red clover	175-321	250
RC-535	Red clover	102-263	178
RC-543	Red clover	146-277	218
A-502	Alfalfa	102-438	257
A-508	Alfalfa	146-438	282
A-512	Alfalfa	190-365	291
A-515	Alfalfa	175-438	295
A-532	Alfalfa	234-409	298
A-538	Alfalfa	263-584	457
A-550	Alfalfa	175-555	374
A-557	Alfalfa	219-496	353
T-503	Trefoil	204-467	262
V-505	Vetch	117-350	197
SC-505	Sweet clover	190-496	350
AC-505	Alsike	117-292	188

[a] Based on 25 pycnidia per isolate.

Twenty monoconidial isolates (8 from alfalfa, 8 from red clover, and 1 from each of vetch, trefoil, alsike, and sweet clover) were grown on 6 different agar media: potato-dextrose (PDA), corn meal, Czapek's, malt, alfalfa extract, and red clover extract. On none of the 6 media could the isolates from alfalfa be distinguished from the isolates from red clover. In addition, isolates from the other legumes were similar to one or more of those from red clover or alfalfa.

Pycnidial development.—Cultures varied greatly in their ability to produce pycnidia in culture. Some of the 137 monoconidial isolates fruited prolifically on PDA, whereas others produced few or no pycnidia



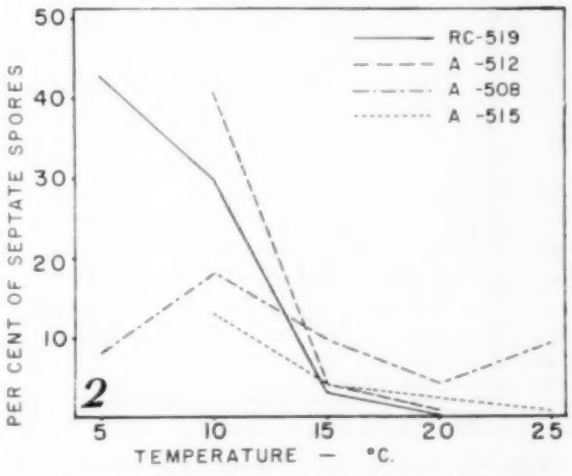


Fig. 1-2. Fig. 1. Spore length (range and average of 50 spores measured per culture) of 20 cultures of blackstem pathogens from different hosts, in μ, when grown 28 days on potato-dextrose agar in Petri plates. **Fig. 2.** The effect of temperature on the percentage of septate spores of 3 isolates from alfalfa and 1 from red clover.

or spores. This was found with other media as well. Sectors from the parent isolates commonly differed in their quantitative production of pycnidia. The quantitative production of pycnidia depended on the particular isolate and the medium. The size of the pycnidia, even within the same isolate, varied considerably when the fungus was grown on PDA (Table 2). The range of the average pycnidial size was higher for cultures from alfalfa than from red clover. However, it was impossible to distinguish 2 cultures such as RC-519 and A-515 on the basis of size of pycnidia. Furthermore, as is presented in another section, the difference in size of pycnidia was not apparent on host tissue. Slight variations in pycnidial color from a tan to black were observed in sectoring colonies. These color differences, though generally very slight, were present in most sectoring colonies, and were the most distinct prior to maturity. The pycnidia were scattered in most cultures; however, some clumping was found in an occasional sector.

Spore size.—A large portion of the spores had a range in length of 4–7 μ, as can be seen in Fig. 1. There was no consistent difference in spore size of isolates from different hosts, except for culture A-502, which had large spores in the first experiment. In later experiments involving subcultures of this isolate, only spores averaging about 5 μ in length were observed. It is therefore concluded that the subcultures with smaller spores were derived from sectors of the original A-502 isolate. This observance of approximately the same range in spore size, irrespective of host, is not in agreement with earlier findings (5, 8). From Fig. 3 and 4 it can be seen that the media on which the cultures were grown had no marked effect on spore size with most cultures; however, practically no change was observed with culture RC-522.

Percentage of septate spores.—Several workers (2, 4, 6, 9, 15, 16, 17, 18) have shown that some isolates from both red clover and alfalfa have septate spores, but that other isolates from both hosts have only non-septate spores. Of 629 mass isolates examined, over 75% of the collections from both red clover and alfalfa had only non-septate spores. Table 3 summarizes the data on the percentage of septate spores in 137 monoconidial isolates grown on PDA. It is obvious from the data that the percentage of septate spores produced by the organism from a given host is not constant, but varies among isolates.

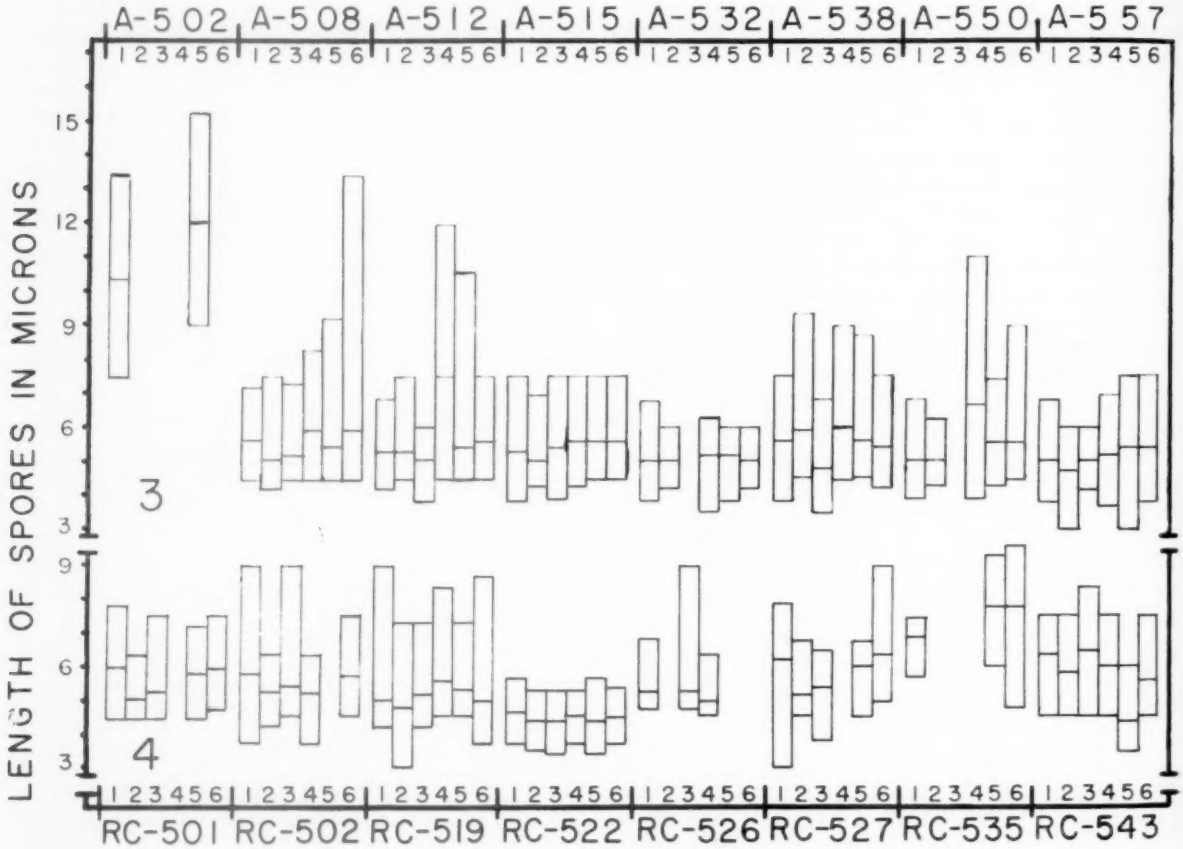


Fig. 3-4. The effect of 6 different media on spore size (range and average of 50 spores measured per culture) of 8 cultures of blackstem pathogens on 3) alfalfa and 4) red clover. Media were: 1, potato-dextrose agar; 2, Czapek's agar; 3, malt agar; 4, corn meal agar; 5, alfalfa agar; 6, red clover agar. Range of spore size: Base of rectangle indicates maximum length; midline is mean; top of rectangle is maximum length.

TABLE 3.—*The number of cultures of blackstem pathogens with their respective percentages of septate spores when grown on potato-dextrose agar*

Host	Percentage of septate spores				
	0	trace to 5	6–20	21–50	51–100
Red clover	45	7	0	2	0
Alfalfa	45	11	2	2	2
Trefoil	3	0	0	0	0
Vetch	4	0	1	0	0
Sweet clover	5	2	0	0	0
Alsike	5	1	2	0	0

For an analysis of stability of cultures to produce a given percentage of septate spores, 117 and 112 monoconidial isolates were obtained, respectively, from an isolate from red clover and an isolate from alfalfa. This particular isolate from red clover had no septate spores, though some of the subisolates did. Six percent of spores from the isolate from alfalfa were septate, and subisolates ranged from 0 to 6% in amount of septate spores.

It is significant to note that a monoconidial isolate from alfalfa with 2% septate spores yielded some monoconidial subisolates that produced no septate spores and others that produced as high as 50% septate spores. The percentage of spores with a septum, therefore is not a valid means for distinguishing between these fungi.

The medium on which the cultures were grown had a decided effect on whether a culture produced septate spores. One isolate from red clover produced no septate spores on PDA, corn meal, alfalfa extract, or red clover extract agar media, whereas 12% of the spores were septate on Czapek's agar.

Only one of 8 isolates from red clover, RC-501, produced any septate spores when grown on autoclaved alfalfa and red clover stems. Seven of 8 isolates from alfalfa produced at least a few septate spores when grown on either red clover or alfalfa stems. These results might indicate a distinction between isolates from red clover and alfalfa, but distinguishing between red clover isolates and an alfalfa isolate such as A-532, which produced no septate spores on either autoclaved red clover or alfalfa stems, does not appear to be possible on host stems.

The effect of temperature at which the cultures were grown on the percentage of septate spores is presented in Fig. 2. One of 8 isolates from red clover and 4 of 8 isolates from alfalfa produced a higher percentage of septate spores at the lower temperature.

Table 4 presents data on the percentage of germinating spores with a septum. Several observations from this experiment were pertinent to the comparative study. First, in all isolates except two from red clover, some septa were produced in the spores when they germinated. Secondly, in cultures that normally produced only a trace of septate spores, as many spores as 45% had one septum. Furthermore, no difference in rate of germination was found between isolates from alfalfa and from red clover.

TABLE 4.—*Germination percentage and percentage of germinating spores of blackstem pathogens that were septate in sterile distilled water*

Host	Culture no.	Per cent germination[a]	Per cent of germinating spores that were septate	
			Range	Average[b]
Red clover	RC–501	55	0–5	1.5
Red clover	RC–502	58	0–4	1.0
Red clover	RC–519	18	0–15	6.3
Red clover	RC–522	28		0.0
Red clover	RC–526	58	0–1	0.2
Red clover	RC–527			20.0 (1 repl.)
Red clover	RC–535	53		0.0 (5 repl.)
Red clover	RC–543	84	0–4	1.5
Alfalfa	A–502	95	65–100	82.5 (2 repl.)
Alfalfa	A–508	37	1–10	6.2
Alfalfa	A–512	32	0–33	10.8 (6 repl.)
Alfalfa	A–515	39	5–15	11.0
Alfalfa	A–532	23	0–5	1.0
Alfalfa	A–538	45	0–45	20.3
Alfalfa	A–550	57	4–20	9.0 (5 repl.)
Alfalfa	A–557	16	0–20	4.0 (6 repl.)
Trefoil	T–503	22	0–3	0.5
Vetch	V–505	37	0–3	0.9
Sweet clover	SC–505	35	0–15	9.2
Alsike	AL–505	26	9–1	0.2

[a] 12 hours following subjection of spores to conditions favorable for germination.

[b] Average of 7 replications unless indicated otherwise.

Morphology of blackstem pathogens on host tissue.—The pycnidia were more uniform in size when produced on infected plant parts collected in the field than when formed on an artificial medium. Their range of diameters on host tissue was 150–250 μ. Pycnidia on infected alfalfa plants were the same size and had the same range in size as pycnidia on infected red clover plants. The pycnidia are produced primarily on dead tissue, and only rarely have pycnidial primordia been observed on what appeared to be living tissue. Immature pycnidia are light to dark brown; mature pycnidia become black and carbonaceous, and are globose, with a short neck and a small ostiole. Little variation in shape or color of pycnidia was observed between collections, regardless of the host from which the collections were obtained.

Johnson and Valleau (5) stated that spores on alfalfa were uniform in size and shape, while spores on red clover were more variable in size. Of 185 collections examined in this study, about one half of the isolates from red clover had spores that were variable in size and shape. Just as high a frequency

TABLE 5.—*Number of collections of host material infected with spring blackstem fungi and the relative percentage of septate spores found in the collections*

Host	Total	Percentage of septate spores			
		0	1–5	6–20	21–50
Alfalfa	96	86	7	2	1
Red clover	89	75	11	3	0

of spores of variable size was observed among collections from alfalfa.

Table 5 presents data on the number of collections of infected host material and the relative percentage of septate spores found in the collections. Clearly, collections of the fungus with septate spores are just as frequent on red clover as on alfalfa.

Growth rate in culture.—When the 137 monoconidial isolates were grown on PDA from uniform mycelial inocula, there were considerable differences in the diameter of different monoconidial lines, but this was not associated with the host from which they were isolated.

Schenck and Gerdemann (17) stated that it was possible to distinguish isolates from alfalfa and red clover on the basis of growth rate from a single spore on PDA. The red clover isolates they studied grew faster than did the isolates from alfalfa.

For tests on whether such a difference was characteristic of the present material, 8 cultures, representing 4 cultures from red clover and 4 from alfalfa, were selected at random. The 4 cultures from red clover were Il-RC-1 and Il-RC-2 (from red clover collected in Illinois) and M-RC-1 and M-RC-2 (from red clover collected in Minnesota). The 4 cultures from alfalfa were Il-A-5, M-A-IE, M-A-2, M-A-3 (the first from alfalfa collected in Illinois and the others from Minnesota).

The data on radial expansion are presented in Fig. 5. Very little difference in culture diameter was observed among cultures derived from single spores obtained from a single monoconidial parent culture. Additional data (2) have shown a considerable range in growth rates among cultures from both alfalfa and red clover.

One obvious conclusion is that not all cultures from red clover grow faster from a single spore than do cultures from alfalfa; however, a higher incidence of fast-growing isolates was obtained from red clover than from alfalfa. There is considerable difference in growth rate from a single spore of cultures from different sources when the growth is measured as radial expansion of cultures on PDA in Petri plates.

Nuclear condition of conidia.—With all the variability among cultures derived from single spores, it was thought necessary to evaluate the nuclear condition of the spores. If the spores were multinucleate, a phenomenon such as heterocaryosis could possibly account for the variability observed. If the spores were uninucleate, such variability would have to arise from mutation or from micro-environmental effects on the expression of a given genotype. A nuclear stain developed by Grasso (3) for the smuts, a modification of the Giemsa-HCl stain, was found to be satisfactory for the blackstem fungi.

Unicellular conidia contained only one nucleus. Septate spores contained one nucleus per cell. The germ tubes of spores generally contain one nucleus per cell other than the terminal cell, which was generally multinucleate. All isolates were similar in nuclear condition, regardless of the host from which they were isolated. Nuclei appeared to be dividing by endomitosis.

The variability observed, therefore, must arise either from mutation or from mirco-environmental effects on the expression of a given genotype.

Hyphal anastomosis.—In a study on the germination of conidia, a few conidia were observed either to fuse directly or to form short hyphal connections, either between germ tubes or between a germ tube and a conidium. This is evidence that anastomosis occurs within a particular isolate. If anastomosis occurs between isolates from alfalfa and from red clover, it would possibly indicate a close relationship between them. Such a relationship has been found in the Koji molds (4). Furthermore, if heterocaryosis is associated with hyphal anastomosis, it would be evidence that isolates from the two hosts draw on a common gene pool. Hyphal anastomoses, though infrequent, were observed when conidia of 4 cultures, 2 from alfalfa and 2 from red clover, were streaked 1 in. apart (in all possible combinations) on agar plates and allowed to grow together. It was not possible to observe anastomosis between conidia or young germ tubes of 2 different cultures, since no way was found of consistently differentiating between the spores of any 2 isolates.

The presence of hyphal anastomosis between 2 isolates is taken in these experiments as evidence of a close relationship of the isolates concerned. Anastomosis occurred as often between isolates from different hosts as between isolates from the same host.

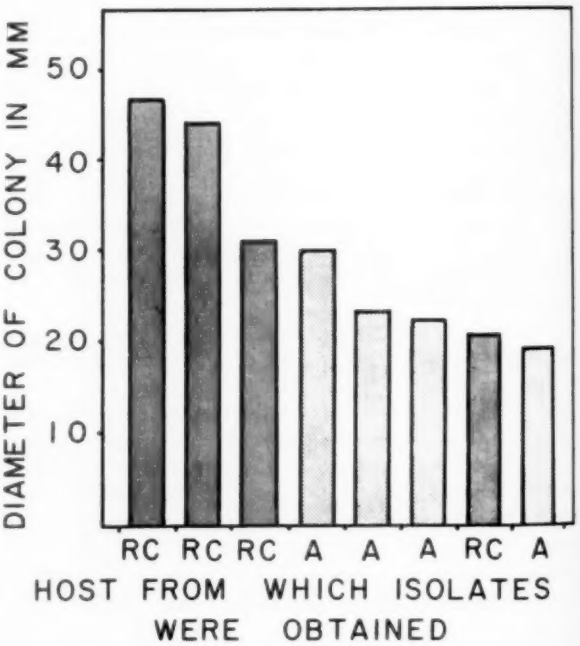


Fig. 5. The diameter of colonies from 8 spore sources when the colony was started from a single spore and allowed to grow 6 days on potato-dextrose agar.

Tests of pathogenicity.—All of the 50 isolates tested were pathogenic on red clover and alfalfa. Some isolates from red clover and some from alfalfa were equally pathogenic on both hosts. The majority of cultures from red clover were more pathogenic on red clover than on alfalfa. The converse was true of isolates from alfalfa.

Considerable variation in pathogenicity was observed between subisolates from 4 culture sources, 2 from red clover and 2 from alfalfa. Considering the uniformity in growth rate of the isolates from a single source, there was great diversity among these same cultures in pathogenicity on alfalfa and red clover. For example, A-612 was very pathogenic on alfalfa and only moderately so on red clover; A-614 was very pathogenic on both alfalfa and red clover; and A-621 was very weakly pathogenic on either host, even though all subisolates came from the same source isolate. All the plants used in these inoculations were of clonal origin so that variability due to the genotype of the host would be eliminated.

Discussion and conclusion.—Several conclusions are evident from the data presented. On the basis of method of development of the pycnidia and range of variability of cultural characteristics, the cultures were essentially identical. The monoconidial isolates were so unstable in respect to appearance, sporulation, and per cent of septate spores that it was common for 3 mycelial transfers from a test tube culture to Petri plates to yield triplicate cultures that were distinctly different.

The range in variability among isolates from alfalfa and red clover was similar. Of the characters studied, none was found to be consistently associated with the host from which the culture was isolated. Percentage of septate spores was a variable character in isolates from both hosts. Not only were isolates variable in the percentage of septate spores produced at any time or in any subculture, but percentage of septate spores could be changed easily, and in some cases greatly, with slight changes in environment. Some of the cultures had a higher percentage of septate spores when grown at 5–15°C than when grown at 25°C. The magnitude of the increase in percentage of septate spores with environmental changes differed with cultures. More of the isolates responding to the temperature changes were from alfalfa than from red clover. However, the greatest magnitude of change in percentage of septate spores was about the same for both red clover and alfalfa isolates. Isolates from alfalfa had the same range in spore size as did isolates from red clover.

Anastomosis occurred as frequently between isolates from both hosts, alfalfa and red clover, as between isolates from the same host. If hyphal anastomosis is an indication of the relationship of isolates, as has been indicated by other workers (4), the observation is further evidence that these fungi represent a single group of highly variable individuals. If heterocaryosis occurs with the hyphal anastomosis, then the evidence is good that the cultures studied draw on a common gene pool.

From the standpoint of pathogenicity or association with host species, there is no essential difference of taxonomic value. Nineteen cultures from alfalfa that were inoculated on red clover infected red clover. Seventeen cultures from red clover that were inoculated on alfalfa infected alfalfa. Furthermore, in many of these tests, the cultures from alfalfa were as virulent on red clover as on alfalfa, and the same was true of isolates from red clover on alfalfa. There was a tendency, however, for a higher frequency of isolates to be more pathogenic on the host from which the isolate was obtained.

The evidence regarding isolates from alsike, sweet clover, vetch, and trefoil is the same. Because there were so few isolates, no definite conclusions are made regarding them. However, the results indicate that they also belong to this group of fungi that are similar to each other on the basis of morphology and physiology in culture, or host relationships.

The question then arises as to what shall be considered the valid name for the organism. If the organism is considered a species of *Ascochyta*, the valid name is *A. imperfecta* Peck. If the organism is considered a species of *Phoma*, *P. herbarum* West. var. *medicaginis* Fckl. is the valid name. The organism is considered to be a member of the genus *Phoma* on the basis that 1) the majority of the cultures studied had only non-septate spores when grown under any particular environmental condition and 2) that 86% of the collections of the fungus on the hosts had only non-septate spores. An additional 10% had only 1–5% septate spores. On its "natural" habitat, the host plants, only 14% of the collections could possibly have been recognized as a species of *Ascochyta*, and several of these had only 1 septate spore of several thousand spores observed in the collection. The organism best conforms to the description of the genus *Phoma* on the basis of predominance of cultures with non-septate spores and the predominance of non-septate spores in those cultures that do have septate spores. Therefore, the writer agrees with Schenck and Gerdemann (17) that the name *Phoma herbarum* West. var. *medicaginis* Fckl. is the valid name for the organism on alfalfa.

Peck described spores of the species *Ascochyta imperfecta* as "variable, continuous to pseudo-uniseptate." The description is considered invalid, because the organism did not belong in the genus *Ascochyta* if it did not have septate spores.

The present data do not support the conclusion of Schenck and Gerdemann (17) regarding the retention of a separate species, *Phoma trifolii*, as the organism causing the spring blackstem disease on red clover. The organism on red clover is indistinguishable morphologically, physiologically, and pathogenically from the organism on alfalfa. Differences between isolates are found; however, these differences are found among isolates from both alfalfa and red

clover. Therefore, they are considered to be only one species.

Since *Phoma herbarum* var. *medicaginis* is the oldest name, it is suggested that the name *Phoma trifolii* E. M. John & Valleau should be listed in synonomy, and it is concluded that *Phoma herbarum* West. var. *medicaginis* Fckl. is the valid name for the organism inciting the spring blackstem disease on red clover and alfalfa.

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ARTHUR J. MIX, 1888-1956

Norvel M. McClung[1]

The death of Professor Arthur Jackson Mix on September 8, 1956, ended the career of one of the most distinguished faculty members at the University of Kansas. Prof. Mix came to that institution as instructor in Plant Pathology in the fall of 1916, and his productive life was largely spent there. He was promoted to Associate Professor in 1919, Professor in 1924, and head of the Department of Botany in 1931, a position he held until 1953 when he resigned the chair to take a trip around the world. On his return he continued a full teaching and research program until his final illness in June, 1956.

The following positions were held and trips taken during leave from the University: 1917-18, Assistant Pathologist, U. S. Dept. of Agriculture; 1929-30, travel, study, and collecting in England, Germany, Switzerland, and France; summer 1930, attendance at the International Botanical Congress at Cambridge, England; 1935, travel and collecting in England, Norway, Sweden, Holland, Belgium, and France, and attendance at the International Botanical Congress in Amsterdam; summer 1939, travel and collecting in Sweden, Lapland, and Norway; 1939-40 study at Farlow Herbarium, Harvard University, and teaching at Cornell University; 1945-46, plant pathologist, U. S. Dept. of Agriculture; 1950, travel and collecting in Norway, Sweden, Germany, Italy, France, and England, and attendance at the International Botanical Congress at Stockholm; 1953-54, travel and collecting in England, Norway, Italy, Canary Islands, Ceylon, India, and Japan.

Thus, Prof. Mix traveled widely and collected specimens of the fungus for which he was an international authority, *Taphrina* species. The story of his experiences during all but the last of these trips is amusingly recounted in his "My Life with *Taphrina*," from a speech given in May, 1952, at the annual banquet as retiring president of the Kansas chapter of Sigma Xi, and published in *Mycologia* in 1958—**50**(3): 315-325.

Prof. Mix was born September 30, 1888, in Bolivar, N.Y., the youngest of the four children of Charles M. and Rose Kenyon Mix. At Hamilton College he was an honor student and was elected to Phi Beta Kappa. He was very interested in public speaking, science, and classical languages. He even contemplated a teaching career in Latin and Greek, but finally decided in favor of science. However, a strong interest remained, as his later language facility showed. He read German, French, Danish, Norwegian, and Swedish with ease, and Dutch and Russian adequately, and spoke German well. After graduation, in 1910, he took a job with a lumber company in Little Current, Ontario.

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There he survived the rigors of a Canadian winter, an epidemic of smallpox in the camp, and the thrill of fishing in Georgian Bay, and returned to the States the next summer, resolved on further education. In the fall he enrolled at Cornell University on a Fellowship for an advanced degree in Plant Pathology. During his graduate career, summers were spent working for the Champlain Valley Fruit Growers Association, where he made the rounds of his clients on a motorcycle known as "Apple Tree Annie." Research for his thesis, "Sun-Scald of Fruit Trees—A Type of Winter Injury," was supervised by Drs. Donald Reddick and H. H. Whetzel. He was granted the Ph.D. degree in February, 1916, and spent 1915-16 as assistant botanist at the New York Experimental Station, Geneva, N. Y.

In his first year at the University of Kansas he met Katharine Lyon, a fellow "refugee" from Cornell. They were married in 1917 at the Lyon home, in Hudson, N. Y. During World War I he served first as a port inspector of fruits and vegetables in New York City, then as a spray specialist at Sanford, Florida, where he furthered the war effort, he later said, by driving the meanest pair of mules in the

[1] The preparation of this manuscript has been made considerably easier by the very generous help of Mrs. A. J. Mix, for which I am grateful, and without which this could not have been written. I have "pirated" freely, with permission, from her.

south. After the Armistice the Mixes returned to Lawrence, and soon bought the house at 1134 Louisiana Street, where they lived until 1955. The Mix home became a sanctuary for foreign students, particularly Scandinavians, and its legendary charm, warmth, and hospitality were extended to many friends from all over the world.

In the second World War Dr. Mix became chairman for the Save the Children Federation in Lawrence, which resulted in the Jayhawk Nursery, near Birmingham, England, for bombed-out English children and orphans. At the end of 1944 he resigned as chairman of this committee and took a war job with the Department of Agriculture's Emergency Plant Disease Survey, finishing the war years as a member of a research team studying cellulose-decomposing fungi at Pennsylvania State University. Returning to the University of Kansas in the fall of 1946, he expanded the Botany Department, adding several new staff members and strengthening graduate offerings.

Professor Mix received many honors during his life: listed in *Who's Who in America*, 1954-57; president of the Kansas Chapter of Sigma Xi, 1950-52; councillor (1948) and Vice President (1952) of the Mycological Society of America; Fellow of the A.A.A.S., 1925; and Honorary Doctor of Science, Hamilton College, 1955.

He was the author of some 40 papers, including a monograph of the genus *Taphrina* and (with the author) *Mycology for Students of Bacteriology and Medicine*. The latter was used for many years in the course in medical mycology taught at the University of Kansas.

Professor Mix became ill in June, 1956, and after several hopeful short convalescent periods, succumbed. He is survived by his wife, Katharine Lyon Mix, who continues to live in the house that he enjoyed planning and seeing built, and lived in happily for a year. This poem, found in his notebook by Mrs. Mix, is a fitting ending to the story of a great mycologist, teacher, investigator, and classicist:

> I would live on through a long October,
> Air drenched with sunlight, warming as old wine,
> Like a drowsing wasp, his honey forage ended,
> Cling to the berry of the aging vine,
> Ripen like fruit in October sunshine,
> Fall as the fruit at October's fall,
> Fall with the loosened leaf slow floating downward,
> Cease with the whisper, "This is all."